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EDITED BY

SIMON FLEXNER, M.D. PEYTON ROUS, M.D.

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# TREATMENT OF EXPERIMENTAL ACUTE GENERAL PERITONITIS IN THE DOG WITH ILEOSTOMY AND SODIUM CHLORIDE SOLUTION.

By THOMAS G. ORR, M.D., and RUSSELL L. HADEN, M.D.

*(From the University of Kansas School of Medicine, Kansas City, Kansas.)*

(Received for publication, November 21, 1928.)

Enterostomy as an aid in the treatment of peritonitis has attracted the attention of many clinicians in recent years. Since reports of its value have been somewhat conflicting we have made a study of the treatment of peritonitis by ileostomy from the experimental standpoint. In a recent publication we have reported the changes which occur in the blood chemistry in experimental general peritonitis (1). The blood chemistry has also been studied in this series of experiments. In addition to the treatment of general peritonitis with ileostomy a small series of dogs has been treated with both ileostomy and 1 per cent salt solution.

Normal dogs, weighing from 7 to 15 kilos, were used in these experiments. Operations were done under complete ether anesthesia with aseptic technic. General peritonitis was produced by ligating the appendix with tape. At the same operation the ileum was divided 6 inches above the cecum, the distal portion invaginated with a purse string suture and the proximal portion brought to the surface through the wound and sutured to the fascia and skin. In some cases a tube was placed in the lumen of the ileum to insure drainage. Blood was drawn daily and sometimes twice daily from the jugular vein for chemical analysis. The animals were kept in a warm room in metabolism cages and given water *ad libitum*.

The non-protein nitrogen was determined by the method of Folin and Wu (2), the urea nitrogen by the Van Slyke and Cullen modification of the Marshall method (3) and the carbon dioxide-combining power by the method of Van Slyke (4). The chlorides were determined on the tungstic acid filtrate as suggested by Gettler (5).

## OBSERVATIONS.

In order to produce a general peritonitis, the appendix was freed at its base and snugly ligated with tape. Any animal developing

TABLE I.

*Appendix Ligated to Produce Peritonitis. Ileum Sectioned 6 Inches above Cecum and Proximal Open End Sutured to Skin for Drainage.*

| Dog No. | Day after operation | Blood | | | $CO_2$-combining power | |
|---|---|---|---|---|---|---|
| | | Amount per 100 cc. | | | | |
| | | Total non-protein nitrogen | Urea nitrogen | Chlorides | | |
| | | *mg.* | *mg.* | *mg.* | *vol. per cent* | |
| 1 | 0 | 34.1 | 18.2 | 510 | 36.2 | Operation |
| | 1 | 48.2 | 25.2 | 460 | 40.0 | |
| | 2 | 91.5 | 47.6 | 390 | 38.1 | Autopsy—general peritonitis |
| 2 | 0 | 27.7 | 12.1 | 440 | 36.2 | Operation |
| | 1 | 31.7 | 13.5 | 420 | 36.2 | |
| | 2 | 94.0 | 52.3 | 350 | — | Autopsy—general peritonitis |
| 3 | 0 | 33.0 | 17.2 | 460 | 34.3 | Operation |
| | 1 | 40.0 | 17.7 | 450 | 34.3 | |
| | 2 | 136.8 | 67.7 | 330 | — | Autopsy—general peritonitis |
| 4 | 0 | 41.6 | 22.4 | 470 | 30.5 | Operation |
| | 1 | 77.0 | 39.2 | 380 | 34.3 | |
| | 2 | 165.0 | 65.3 | 370 | — | Autopsy—general peritonitis |
| 5 | 0 | 24.8 | 14.4 | 430 | 36.2 | Operation |
| | 1 | 20.2 | 9.8 | 400 | 34.3 | |
| | 2 | 41.0 | 19.6 | 380 | 38.1 | |
| | 3 | 50.8 | 17.7 | 400 | 36.2 | |
| | 4 | 107.0 | 66.3 | 370 | 28.7 | |
| | 5 (a.m.) | 208.0 | 91.0 | 320 | 29.6 | |
| | 5 (p.m.) | 190.0 | 83.5 | 330 | 20.2 | Autopsy—general peritonitis |
| 6 | 0 | 26.8 | 12.1 | 460 | 31.5 | Operation |
| | 1 (a.m.) | 59.5 | 37.3 | 270 | — | |
| | 1 (p.m.) | 104.0 | 51.3 | 320 | 29.0 | |
| | 2 (a.m.) | 131.0 | 74.7 | 290 | 30.5 | |
| | 2 (p.m.) | 114.0 | 80.7 | 300 | 37.2 | |
| | 3 | 159.0 | 80.7 | 280 | 40.9 | Autopsy—general peritonitis |
| 7 | 0 | 28.5 | 14.5 | 460 | 34.3 | Operation |
| | 1 | 42.8 | 17.8 | 410 | 41.9 | |
| | 2 | 115.0 | 59.8 | 400 | 32.4 | |
| | 3 (a.m.) | 140.0 | 77.1 | 330 | 36.2 | |
| | 3 (p.m.) | 254.0 | 129.8 | 290 | — | Autopsy—general peritonitis |

TABLE I—*Concluded.*

| Dog No. | Day after operation | Blood | | | $CO_2$-combining power | |
|---|---|---|---|---|---|---|
| | | Amount per 100 cc. | | | | |
| | | Total non-protein nitrogen | Urea nitrogen | Chlorides | | |
| | | *mg.* | *mg.* | *mg.* | *vol. per cent* | |
| 8 | 0 | 57.8 | 31.7 | 500 | 37.2 | Operation |
| | 1 | 39.5 | 20.5 | 470 | 38.1 | |
| | 2 | 70.0 | 36.8 | 390 | 36.2 | |
| | 3 | 61.0 | 28.0 | 350 | 43.8 | |
| | 4 | 63.0 | 29.8 | 340 | 44.7 | |
| | 5 | 88.3 | 35.9 | 350 | 34.3 | |
| | 6 | 148.0 | 94.8 | 260 | — | Autopsy—general peritonitis |
| 9 | 0 | 28.0 | 8.8 | 530 | 43.8 | Operation |
| | 1 | 23.3 | 11.2 | 500 | 30.5 | |
| | 2 (a.m.) | 59.0 | 32.2 | 410 | 22.1 | |
| | 2 (p.m.) | 107.0 | 56.0 | 410 | 29.6 | |
| | 3 | 103.0 | 63.0 | 350 | 22.1 | Autopsy—general peritonitis |

complications was discarded. Autopsy was done in every case to determine the extent of peritoneal involvement. More than 50 per cent of the dogs developed a localized abscess at the site of the appendix or got well. Only those having a definite general peritonitis are recorded here.

In the first series, which included nine animals (Table I), the length of life varied from 2 to 6 days with an average of 3 1/9 days. In a previous study of eight animals with general peritonitis without drainage of the ileum (1) the average length of life was 4 1/8 days.

The changes in the chemistry of the blood were in all cases similar to those previously found in general peritonitis, showing an increase in the non-protein and urea nitrogen, a decrease in the chlorides and no constant change in the carbon dioxide-combining power.

The second series of animals with ligated appendix and ileostomy (Table II), was treated early with 40 cc. of 1 per cent sodium chloride solution per kilo of body weight. These dogs lived from 4 to 18 days or an average of 10 1/3 days. With the administration of salt solution, the changes in the chemistry of the blood were not marked. In some

TABLE II.

*Appendix Ligated to Produce Peritonitis. Ileum Sectioned 6 Inches above Cecum and Proximal Open End Sutured to Skin. Treatment with 1 Per Cent Sodium Chloride Solution.*

| Dog No. | Day after operation | Blood | | | $CO_2$-combining power | |
|---|---|---|---|---|---|---|
| | | Amount per 100 cc. | | | | |
| | | Total non-protein nitrogen | Urea nitrogen | Chlorides | | |
| | | *mg.* | *mg.* | *mg.* | *vol. per cent* | |
| 1 | 0 | 29.1 | 9.3 | 410 | 31.5 | Operation, 400 cc. 1% NaCl |
| | 1 | 31.6 | 9.8 | 480 | 36.2 | " " " " |
| | 2 | — | — | — | — | " " " " |
| | 3 | 38.0 | 14.9 | 540 | 21.9 | " " " " |
| | 4 | 21.1 | 9.3 | 530 | 29.6 | " " " " |
| | | | | | | Autopsy—general peritonitis |
| 2 | 0 | 54.6 | 35.0 | 530 | 31.5 | Operation, 408 cc. 1% NaCl |
| | 1 | 38.9 | 14.0 | 490 | 30.5 | " " " " |
| | 2 | 22.5 | 7.0 | 470 | 30.5 | " " " " |
| | 3 | 22.9 | 7.9 | 470 | 29.6 | " " " " |
| | 4 | 27.0 | 9.3 | 460 | 34.3 | " " " " |
| | 5 | 20.0 | 7.5 | 430 | 32.4 | " " " " |
| | 6 | 27.0 | 14.0 | 480 | 31.5 | " " " " |
| | | | | | | Autopsy—general peritonitis |
| 3 | 0 | 23.3 | 9.8 | 410 | 24.0 | Operation, 400 cc. 1% NaCl |
| | 1 | 24.6 | 14.4 | 420 | 29.6 | " " " " |
| | 2 | 24.0 | 11.6 | 460 | 32.4 | " " " " |
| | 3 | 21.4 | 14.0 | 490 | 32.4 | " " " " |
| | 4 | 22.4 | 14.9 | 500 | 22.1 | " " " " |
| | 5 | 22.8 | 8.8 | 540 | 25.6 | " " " " |
| | 7 | 20.7 | 8.8 | 550 | 25.6 | " " " " |
| | 8 | 20.4 | 9.3 | 580 | 27.5 | " " " " |
| | 9 | 21.1 | 7.0 | 580 | 28.5 | " " " " |
| | 10 | 22.5 | 9.3 | 630 | 21.1 | " " " " |
| | 11 | 20.7 | 10.7 | 610 | 16.4 | " " " " |
| | 12 | 22.9 | 8.4 | 600 | 18.3 | " " " " |
| | 14 | 23.0 | 7.9 | 620 | 22.1 | " " " " |
| | 16 | 30.0 | 9.8 | 650 | 14.5 | " " " " |
| | 18 | 41.6 | 36.4 | 890 | | " " " " |
| | | | | | | Autopsy—general peritonitis |

TABLE II—*Concluded.*

| Dog No. | Day after operation | Blood | | | $CO_2$-combining power | |
|---|---|---|---|---|---|---|
| | | Amount per 100 cc. | | | | |
| | | Total non-protein nitrogen | Urea nitrogen | Chlorides | | |
| | | *mg.* | *mg.* | *mg.* | *vol. per cent* | |
| 4 | 0 | 24.2 | 17.2 | 440 | 32.4 | Operation, 380 cc. 1% NaCl |
| | 1 | 25.2 | 14.9 | 460 | 14.5 | " " " " |
| | 3 | 21.6 | 12.1 | 480 | 29.6 | " " " " |
| | 4 | 20.8 | 11.2 | 510 | 32.4 | " " " " |
| | 5 | 20.7 | 13.5 | 430 | 27.5 | " " " " |
| | 6 | 19.0 | 9.8 | 570 | 25.6 | " " " " |
| | 7 | 20.3 | 8.9 | 430 | 21.1 | " " " " |
| | 8 | 41.0 | 18.9 | 460 | 22.1 | " " " " |
| | | | | | | Autopsy—general peritonitis |
| 5 | 0 | 51.8 | 26.1 | 450 | 31.5 | Operation, 352 cc. 1% NaCl |
| | 1 | 28.9 | 18.2 | 480 | 36.2 | " " " " |
| | 2 | 20.3 | 17.2 | 480 | 36.2 | " " " " |
| | 3 | 20.7 | 14.0 | 470 | 37.2 | " " " " |
| | 4 | 21.4 | 11.6 | 430 | 38.1 | " " " " |
| | 5 | 22.2 | 12.1 | 490 | 32.4 | " " " " |
| | 6 | 20.7 | 10.2 | 480 | 17.4 | " " " " |
| | 7 | 20.5 | 11.2 | 510 | 25.8 | " " " " |
| | 8 | 22.9 | 10.2 | 580 | 15.5 | " " " " |
| | 10 | 23.0 | 11.2 | 550 | 16.4 | " " " " |
| | 12 | 30.9 | 14.9 | 550 | 17.4 | " " " " |
| | 13 | 28.5 | 15.8 | 530 | — | " " " " |
| | 14 | 79.8 | 41.1 | 420 | — | " " " " |
| | | | | | | Autopsy—general peritonitis |
| 6 | 0 | 30.9 | 11.6 | 480 | 32.4 | Operation, 280 cc. 1% NaCl |
| | 1 | 34.9 | 10.2 | 410 | 29.6 | " " " " |
| | 2 | 30.9 | 11.2 | 420 | 35.3 | " " " " |
| | 3 | — | — | — | — | " " " " |
| | 4 | 31.9 | 13.0 | 470 | — | " " " " |
| | 5 | 27.7 | 12.6 | 490 | 36.2 | " " " " |
| | 6 | 25.7 | 14.0 | 540 | 27.5 | " " " " |
| | 7 | 23.4 | 11.6 | 510 | 32.4 | " " " " |
| | 8 | 28.2 | 16.3 | 560 | 31.5 | " " " " |
| | 9 | 23.8 | 15.4 | 500 | 32.4 | " " " " |
| | 10 | — | — | — | — | " " " " |
| | 11 | 53.6 | 32.2 | 470 | — | " " " " |
| | 12 | 52.0 | 78.4 | 490 | 11.1 | " " " " |
| | | | | | | Autopsy—general peritonitis |

cases there was a terminal rise in the non-protein and urea nitrogen and in one case, living 18 days, a definite rise in the chlorides. The carbon dioxide-combining power showed no constant change.

## DISCUSSION.

In this study of general peritonitis, treated with ileostomy from the beginning, no beneficial results are noted. Life is not prolonged by this method of treatment nor are the typical changes in the blood chemistry noted in experimental general peritonitis prevented.

The animals treated with 1 per cent solution of sodium chloride hypodermically in addition to ileostomy lived three times as long as those having no salt solution. The administration of the sodium chloride solution was quite effective in preventing the chemical changes of the blood noted in animals without such treatment. The results of treatment with sodium chloride in this study are similar to those previously recorded in the treatment of high intestinal obstruction (6).

## CONCLUSION.

1. In experimentally produced general peritonitis drainage of the gut by ileostomy 6 inches above the cecum has no beneficial effect.

2. Animals with experimentally produced general peritonitis treated with ileostomy plus 1 per cent sodium chloride solution live three times as long as those not given the salt solution.

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# THE RAPID SHALLOW BREATHING RESULTING FROM PULMONARY CONGESTION AND EDEMA.

By EDWARD D. CHURCHILL, M.D., and OLIVER COPE, M.D.

(*From the Surgical Laboratories, Massachusetts General Hospital, Boston.*)

(Received for publication, December 12, 1928.)

Experimental studies by Dunn (1) demonstrated that multiple embolism of the pulmonary arterioles and capillaries is followed by a temporary inhibition of respiration succeeded by rapid shallow breathing. Dunn attributed this phenomenon to the stimulation of sensory nerve endings in the lungs. This explanation was favored by the fact that the injection of starch produced no alteration in the respiratory rate when both vagi had been previously sectioned.

Attracted to this problem by its bearing upon the occurrence of rapid and shallow respiration in lobar pneumonia, Binger and Moore and their associates (2–6) have carried out a carefully planned series of experiments. Their studies chiefly concern the effects of the embolism of pulmonary arterioles and capillaries produced by the intravenous injection of starch grains under varying conditions. These observations were supplemented by other experimentally induced modifications of the pulmonary circulation. In their conclusion (6) to this instructive series of papers they state: "Since rapid and shallow breathing is not the result of (1) anoxemia, (2) increased $pCO_2$ and hydrogen ion concentration of the serum, (3) restriction of pulmonary vascular bed by nearly half, (4) increase in resistance to the flow of blood to and from the lungs, (5) the presence of starch grains in the lungs acting as a local irritant, it must be the result of the secondary pathological changes which occur in the pulmonary parenchyma following embolism. The nature of these changes, congestion and edema has been discussed elsewhere. Whether they operate directly on nerve endings or through their influence on lung volume and tissue elasticity is not certain." In their discussion the authors are inclined toward the hypothesis of direct irritation of the vagal nerve endings,

but wisely decline to theorize in the absence of more direct experimental evidence bearing on this point.

During the course of an investigation having quite another bearing, we have made certain observations which appear to demonstrate the occurrence of the usual signs of vagal stimulation in association with the experimental production of pulmonary congestion and edema. As a method completely different from those of Dunn or Binger was being employed, it seems desirable to briefly recount the results of our experiments.

### *Method.*

A Drinker heart preparation (7) was made in a cat anesthetized with intravenous sodium-barbital (5 per cent solution). This procedure when complete leaves the heart exposed in a normally breathing animal, and is easily made by suturing the edges of the pericardium to the margins of an oval window created in the anterior wall of the chest by the removal of the sternum. After the closure of the chest and the resumption of normal respiration, ligatures were placed about the right branch of the pulmonary artery, and the group of right pulmonary veins respectively. The artery is readily reached from within the pericardium in that portion of its course which lies between the ascending aorta and the superior vena cava. The placing of a ligature about the right pulmonary veins is a more delicate procedure, as the thinness of their walls makes the liability to accidental puncture very real.

After both ligatures were in place they were tied so as to completely occlude the vessels, the one around the artery being tied first in order to avoid the effects of venous obstruction. An especially designed glass cannula was then inserted into the right branch of the pulmonary artery, distal to the point of ligation, and secured in place with a second ligature about the vessel. The cannula was connected by transparent rubber tubing to a glass reservoir which could be raised and lowered to effect changes in pressure. A T-tube leading from a point near the cannula was connected with a mercury manometer to record the pressure. The reservoir and tubing up to the cannula were filled with freshly drawn, heparinized blood from another animal, kept at body temperature.

Respiratory movements were recorded by a thread attached to the costal margin, moving a writing lever on the kymograph record. Systemic blood pressure was recorded by a citrated cannula in the right carotid artery. The blood pressure tracing served also to record pulse rate.

In such a preparation, the right lung is isolated from the rest of the circulation of the animal except for the amount of blood that may enter it through the bronchial artery. Ventilation of this lung is maintained by the normal respiratory movements of the animal as the lung is in its normal position in the closed pleural cavity. By changing the height of the reservoir connected to the cannula in the

right branch of the pulmonary artery, it is possible to alter the intravascular pressure in this isolated lung. Such changes may be produced without directly modifying the dynamics of the systemic circulation, or altering the gaseous exchange in the circulating blood. The latter is maintained by the normally functioning left lung and has been shown in other studies (8) to be adequate for the immediate needs of the animal.

## EXPERIMENTS.

The following experiment illustrates the characteristic changes which have been observed in the respiratory rate and depth and in the systemic blood pressure and pulse rate following a sudden elevation of the intravascular pressure of the isolated right lung. As explained above the complete exclusion of this lung from the circulation of the animal assures that these effects are not due to pressure changes in the heart, variation of blood flow or alteration in the respiratory exchange.

*Protocol. Experiment 1. Feb. 24, 1928. Circulatory Isolation of the Right Lung with the Production of Intravascular Pressure Changes.*—2:30. Cat (2.7 kilos). Anesthesia induced by chloroform-ether to permit the injection of 19 cc. of sodium-barbital (5 per cent solution) into the saphenous vein. 3:08. Artificial respiration temporarily employed during the period when the chest is open. 3:45. Chest closed and residual air withdrawn from the pleural cavities. Artificial ventilation discontinued. 3:48. Blood pressure cannula inserted into the right carotid artery. 4:05. Ligatures placed about the right branch of the pulmonary artery and the right set of pulmonary veins. 4:20. Ligatures tied. Cannula inserted into the right branch of the pulmonary artery, and connected with a pressure reservoir filled with heparinized cat blood. 4:26. (Kymograph record, Fig. 1.) Reservoir elevated. 4:27. Reservoir lowered. 4:28. Reservoir elevated. 4:29. Reservoir lowered. 4:31. (Kymograph record, Fig. 2.) Reservoir elevated. 4:32. Reservoir lowered. 4:33. Experiment concluded. The heart showed no dilatation or change in appearance during the course of the experiment.

*Autopsy.*—No air in pleural cavities. The posterior and dependent parts of the lower lobe of the right lung were edematous. The rest of the lung was congested. A very small area of atelectasis was present in the right upper lobe. The left lung was of normal appearance and downy texture.

Sections of the kymograph tracing of this experiment are shown in Figs. 1 and 2. The results of the first elevation of the reservoir connected with the right pulmonary artery are recorded in Fig. 1. The rise in pressure is recorded in the change of level of line *D*, which indicates an elevation from 0 to 42 mm. of mercury. Following a latent period of 6 seconds, the systemic blood pressure (*A*) slowly

FIG. 1. Section of kymograph tracing from Experiment 1. *A*, systemic blood pressure in mm. of mercury recorded from right carotid artery. *B*, blood pressure base line. *C*, respiratory excursion of costal margin; down stroke of lever on inspiration. *D*, pressure in right branch of the pulmonary artery measured in mm. of mercury. *E*, time in 1 second intervals. *F*, signal marker indicating points when reservoir attached to the cannula in the right branch of the pulmonary artery was raised and lowered. The elevation of intravascular pressure in the isolated lung is followed by an apneic pause of respiratory excursions, succeeded by rapid shallow breathing. There is a simultaneous fall in blood pressure with slowing of the heart rate.

drops from 78 mm. of mercury to 60 mm. The pulse rate diminishes from 240 per minute to 204 per minute. The tracing of the respiratory excursions shows marked changes; a period of apnea lasting 8 seconds is followed by rapid and shallow respirations, the rate increasing from 47.5 to 75 per minute. These effects tended to disappear even before the reduction of pressure, although at the

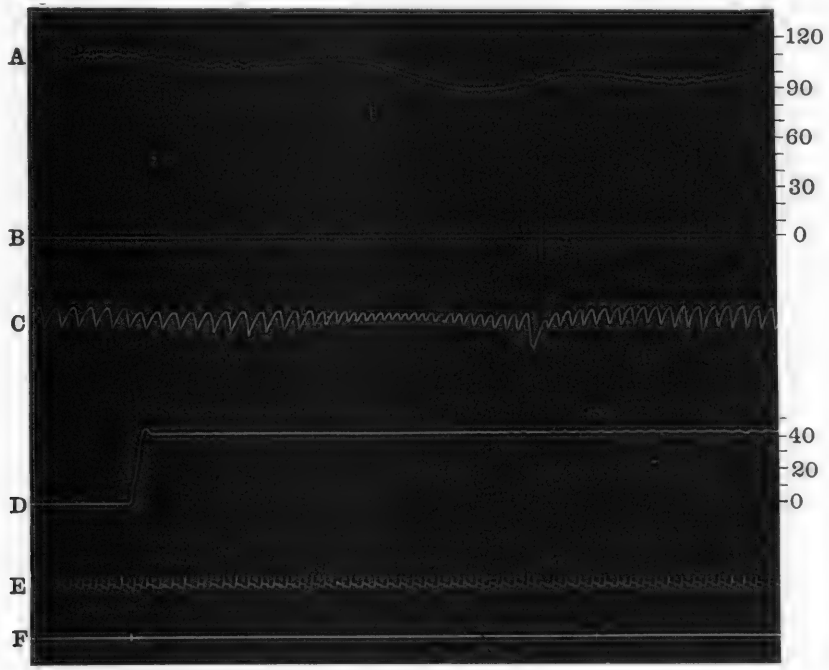


FIG. 2. Section of kymograph tracing from Experiment 1. Tracings as in Fig. 1. The elevation of pressure in the right branch of the pulmonary artery is followed by the institution of rapid shallow breathing without a period of apnea. The fall in blood pressure and slowing of heart rate is as in Fig. 1.

start of the second elevation, the respiratory rate was still increased over its previous value.

In the second experimentally produced rise in pressure, the tracing of which is not shown, similar changes took place. The pressure in the right pulmonary artery was raised from 0 to 38 mm. of mercury. Following a latent period of 7 seconds, the heart rate decreased from 240 to 228 per minute. The systemic blood pressure fell from 82 mm. of mercury to 77. The respiratory rhythm showed

no apneic pause, but the rate was increased from 45 to 80 per minute and the excursions became very shallow.

The third period is shown in Fig. 2. A rise in pressure of 42 mm. of mercury in the right pulmonary artery was followed by a latent period of 13 seconds. The systemic blood pressure dropped from 110 to 90 mm. of mercury, with a coincident slowing of cardiac rate from 252 to 228 contractions per minute. The respiratory rate increased from 45 to 90 per minute and became very shallow. As before, there was a tendency for the respiration to return to its original rate and depth before the pressure was reduced.

This experiment has been repeated fifteen times on six different cats with perfectly consistent results, *viz.*, a slowing of cardiac rate, a fall in systemic blood pressure and the institution of rapid shallow breathing, at times preceded by an apneic pause. It is unusual to be able to repeat the procedure in an individual animal with the same degree of success as was done in the experiment just described. Even here, the return to initial conditions between the changes in pressure was not complete. This is not surprising, as the congestive changes in the lung are presumably of a nature to produce a prolonged effect and not to permit of recovery during the period of the experiment.

## DISCUSSION AND SUMMARY.

These experiments record the effects of the experimental production of pulmonary congestion and edema in a lung completely isolated from the general circulation, but with an intact nerve supply. The resulting changes are: a slowing of the heart rate, a fall in systemic blood pressure and a temporary inhibition of respiration succeeded by rapid shallow breathing. The pulse rate and blood pressure show a rapid and spontaneous return to initial conditions. The respirations show a partial but not a complete return to their former rate and depth. The effects on respiration are similar to those described by Dunn and Binger and Moore which follow multiple embolism of the pulmonary circuit with starch granules. The alterations in the pulse rate and blood pressure are characteristic of the effects of vagal stimulation. A chemical effect on the respiratory center is excluded by the nature of the preparation.

These results, therefore, add further evidence to support the hypothesis that the rapid shallow breathing attending congestion and edema of the lungs is due to the stimulation of nerve endings in the lungs.

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# NON-HEMOLYTIC STREPTOCOCCI AND ACUTE RHEUMATIC FEVER.

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The association of specific non-hemolytic streptococci, of both the alpha and the gamma types, with acute rheumatic fever has been emphasized by several investigators in the past few years. The alpha type has been championed by Clawson (1), who was able to recover the organisms from the blood stream in a relatively high percentage of cases. Streptococci of the gamma type have been described by Small (2) and by Birkhaug (3), chiefly in relation to their presence in the throats of patients with acute rheumatic fever; with both investigators, however, their attention was originally directed to the organisms by the fact that they were recovered from the blood.

Granted that the etiologic factor of acute rheumatic fever were definitely known, it seems reasonable to assume that at least some advances might be made in the treatment of a disease in which, so far, therapeusis has been chiefly symptomatic and empirical. If streptococci of the alpha type are recovered fairly consistently from the blood stream in cases of rheumatic fever, or, if streptococci of the gamma type are occasionally found in the blood and are usually present in the throat, provided, in the latter case, that they do not occur in normal throats, either fact would furnish a lead worthy of extensive investigation.

Early in the fall of 1927 it was decided to take cultures from all available cases of acute rheumatic fever, and this has been done on 25 cases. The methods used have been such as to combine those of Clawson, Small and Birkhaug. Hitchcock (4) has recently published a paper which covers essentially the same ground in regard to the presence of non-hemolytic streptococci of the gamma type in the throats of rheumatic and non-rheumatic individuals.

## *Methods.*

### *Cases.*

The cases of acute rheumatic fever which were studied were carefully selected. Cultures were taken only from cases showing fever with a temperature above 100°F., manifestations of acute involvement of one or more joints and, as a rule, at least a moderate degree of leucocytosis. In those cases which had not received previous medical attention, efforts were made to obtain cultures prior to the administration of salicylates. The majority of the patients were in the wards of the Boston City Hospital, but cultures were obtained from 5 and 2 patients, respectively, through the kind cooperation of the Medical Services of the Massachusetts General and Peter Bent Brigham Hospitals.

### *Primary Cultures.*

*Blood.*—The blood was withdrawn from an arm vein and the culture media inoculated at the bedside. As a rule, 50 to 60 cc. were withdrawn, though in several instances only 25 or 30 cc. were obtained.

One or 2 flasks containing 100 cc. of 0.2 per cent dextrose beef infusion broth (pH 7.5–7.7) were inoculated with 5.0 cc. of blood and 3.0 cc. were added to 50 cc. of beef infusion agar (pH 7.5–7.7), previously melted and cooled to ±42°C. From the latter, after thorough mixing, 3 plates were poured. The remainder of the blood (10 to 40 cc.) was added to 1 or 2 sterile test-tubes and allowed to clot. The supernatant serum was pipetted off and saved for subsequent tests, and each clot was added to a flask containing 200 cc. of 0.2 per cent dextrose beef infusion broth (pH 7.5–7.7).

The blood agar plates were examined daily and discarded, in the absence of growth, at the end of 5 days. The flasks containing the blood and blood clot were examined daily during the 1st week for clouding of the medium or hemolysis. Smears were made and stained by the Gram method at weekly intervals. At the end of a month subcultures were made on blood agar plates, and, in the absence of growth, the flasks were discarded.

*Throat.*—Two swabs were used. Each was rubbed vigorously over all visible portions of the throat posterior to the anterior pillars of the tonsils. Each swab was rubbed, while twisting, over an area approximately 1.0 by 4.0 cm. on the surface of a blood agar plate (5 per cent rabbit's blood in beef infusion agar—pH 7.5–7.7). Proper dilutions were made on the remainder of the plate by smearing from this area with a sterile platinum loop.

*Urine and Stool.*—In several of the cases first studied cultures were made from fresh samples of urines and stools. The former were plated, undiluted, on blood agar plates. With the latter, a small bit was shaken up in a tube containing 0.2 per cent dextrose beef infusion broth, a loopful was spread on the surface of a blood agar plate and 2 more plates were made from the first by smearing with a sterile platinum loop. Suspicious colonies were transferred to dextrose beef infusion broth.

*Procedure.*

All blood or clot culture flasks, which showed growth, were subcultured to blood agar plates and to dextrose beef infusion broth.

With throat cultures, as a rule, 8 suspicious colonies were picked and transferred to a blood agar plate. Those producing characteristic colonies in pure culture after 24 hours incubation were transferred to dextrose beef infusion broth. All strains proving to be streptococci were then transferred to 1.0 per cent lactose beef infusion broth containing phenol red and were also tested for agglutination with rabbit antisera prepared with representative strains of the Small and Birkhaug types of non-hemolytic streptococci.

Gamma type streptococci which differed only in their ability to ferment lactose were frequently recovered from the same throat. Representatives of both the lactose-fermenting and non-lactose-fermenting types were always saved. Duplicate strains were discarded. More extensive sugar fermentation tests and agglutination tests were eventually carried out. When warranted, agglutinin absorption tests were done. The agglutinin content of the patients' sera for autogenous and, sometimes, for various other strains was determined. All strains were tested for the production of a skin-reacting soluble toxin. A more detailed account of the methods follows:

*Morphology.*—Smears were made and stained by Gram's method from 24 to 48 hour cultures in dextrose broth.

*Cultural Characteristics.*—Notes were made concerning surface colonies on blood agar plates and on the type of growth in dextrose broth. All strains were transferred to tubes of beef infusion broth (pH 7.8) containing 1.0 per cent dextrose, lactose, saccharose, mannitol, salicin, inulin or raffinose, together with suitable amounts of phenol red; final readings as to fermentation were taken after 5 days incubation.[1]

*Agglutination.*—Antisera were prepared by injecting rabbits intravenously at 4 or 5 day intervals with 24 or 48 hour cultures of the desired organisms in dextrose broth. The usual dosage was 1, 5, 10, 10, 10 and 10 cc. After an interval of 7 or 8 days, a test bleeding was taken, and, if a sufficiently high titer was obtained, the animal was bled. Otherwise, the injections were repeated.

For the test, 0.5 cc. of a 24 or 48 hour dextrose broth culture was added to an equal amount of the proper dilution of the antiserum. Many of the strains were tested with increasing dilutions starting with a final serum dilution of 1:80. The majority, however, were tested with a single dilution sufficiently high to rule out "group agglutination." Controls with normal salt solution and anti-*B. paratyphosus* B rabbit serum were always set up. The tubes were placed in a water

[1] It had been previously determined that the amount of sugar normally present in beef infusion broth was insufficient to bring about, by its fermentation, significant change of the indicator. This obviated the necessity of using sugar-free broth.

bath at ±40°C. for 24 hours. One plus represented definite agglutination when viewed with a hand lens and 4 plus complete agglutination with a crystal-clear supernatant fluid.

*Agglutinin Absorption.*—48 hour cultures of the desired organisms in 50 cc. dextrose broth were centrifuged at about 3000 revolutions per minute for 40 minutes—no attempt was made to standardize the number of organisms except to discard any culture showing poor growth. The clear supernatant fluid was discarded and 5.0 cc. of a 1:160 dilution of the desired antiserum added. The tubes were incubated at 37.5°C. for 3 hours with frequent shaking and left at room temperature (±22°C.) for 21 hours. They were then centrifuged and the clear supernatant fluid pipetted away. The homologous organism, in 0.5 cc. amounts of a dextrose broth culture, was added to equal amounts of increasing dilutions of the absorbed antiserum. These tubes were subsequently treated like the agglutination tests. With each antiserum and with every series of tests, control tubes of unabsorbed serum and of serum absorbed with the homologous organism were always carried through.

*Agglutinins in Patients' Sera.*—Increasing dilutions of each patient's serum were prepared. Equal amounts (0.5 cc.) of all autogenous strains and various other representative strains were added from 24 to 48 hour dextrose broth cultures. The lowest final dilution of the serum was 1:40. Control tubes with normal salt solution and a non-agglutinating normal serum were made. All tubes were subsequently treated like the agglutination tests. Control tests with sera from non-rheumatic patients were carried out.

*Toxin Production.*—All strains were grown for 10 days in 50 cc. amounts of modified Douglas tryptic digest broth, as described by Birkhaug (3). The cultures were centrifuged and the supernatant fluid filtered through Berkefeld N or V candles. The filtrates were tubed and kept in the ice box. No phenol or tricresol was added. Sterility tests were always carried out. For testing, 1:10 dilutions were made with sterile salt solution and these were injected intradermally in 0.1 cc. amounts in an individual known to be skin-positive to similar amounts of Birkhaug's standard test toxin.[2]

## RESULTS.

*Stock Strains.*—A number of strains of non-hemolytic streptococci from cases of rheumatic fever were obtained through the kindness of Dr. James C. Small of Philadelphia and Dr. Konrad E. Birkhaug of Rochester, New York. The 2 strains obtained from the former were labeled "A" and "B," and from now on will be referred to as "Small A" and "Small B."[3] The 7 strains from the latter were labeled "1b,"

[2] This toxin (1:100 dilution, Lot 3, July 5, 1927) was obtained through the kindness of Dr. Konrad E. Birkhaug of Rochester, New York.

[3] "Small A" and "Small B" correspond to "R1" and "R9" in Small's original paper (2).

"2," "10a," "17," "36," "84" and "167," and will be referred to as "Birk. 1b," "Birk. 2" and so forth.

Morphologically and culturally these 9 strains were practically identical. In broth they all produced a rather heavy ropy sediment with a relatively turbid supernatant fluid. On smear they appeared as rather small Gram-positive diplococci with numerous short to moderately long chains. On blood agar the colonies were round (maximum 2.0 to 3.0 mm. in diameter), tent-shaped, soft, moist, translucent and grayish in color, and they were readily picked out in a mixed culture by a peculiar reddish brown color apparent with transmitted light. There was no change in the color of the surrounding medium except a rather diffuse paling, which occurred after a number of days at ice box temperature. All strains fermented dextrose, saccharose, lactose, raffinose, salicin and inulin, but failed to ferment mannitol.

A number of rabbits were injected intravenously with varying amounts of 24 hour cultures of certain of these streptococci in dextrose broth. For producing monovalent agglutinating sera 3 rabbits were injected with living cultures of Small A, Small B and Birk. 2, respectively. They received injections of 1, 5, 10, 10, 10 and 10 cc. at 4 to 5 day intervals. All 3 failed to show any rise in temperature and the loss in weight was negligible. The first 2 were killed 10 days after the last injection. One (Small A), at autopsy, was negative except for the lungs, which were distended, firm and cut with increased resistance. The other (Small B) showed identical pulmonary changes and, in addition, several firm to friable vegetations on the wall of the left auricle and on the papillary muscles of the tricuspid valve. The third rabbit (Birk. 2) died 2 days after the last injection. The autopsy was negative except for pulmonary changes like those present in the other 2 rabbits.

Two rabbits were given larger amounts of 2 of the cultures—Small A, 10, 20, 18, 20 and 18 cc. and Small B, 10 and 20 cc. at 4 to 5 day intervals. The Small A rabbit was found dead the morning following the last injection. At autopsy, there were friable vegetations on the papillary muscles of the tricuspid valve. The lungs were enlarged and firm. Cultures taken of these vegetations and the heart's blood yielded pure cultures of non-hemolytic streptococci. The Small B

rabbit died 19 hours after the last injection. The autopsy and cultures of the heart's blood were negative. Two more animals received still larger doses—Small A, 23 and 50 cc. and Small B, 23 and 50 cc. In each instance the desired amount of culture was centrifuged at about 3000 revolutions per minute for 30 to 40 minutes, and the sediment resuspended in about 10 cc. of sterile salt solution. The second injections were given the 2nd day following the first. The Small A rabbit was found dead the morning following the second injection. The autopsy was negative, but the blood cultures were positive. The Small B rabbit died on the 5th day following the second injection; the autopsy and blood culture were negative. Two more rabbits received larger doses of heat-killed (100°C. for 1 hour) cultures—Small A, 50 and 50 cc. and Small B, 50 and 50 cc. These were centrifuged and the organisms resuspended in 10 cc. of sterile salt solution. The second injections were given on the 4th day following the first. Both animals gained in weight and failed to show any harmful effects.

Careful inspection of all leg joints, both during life and at autopsy, failed to show the slightest evidence of any sort of joint lesion in any of the animals. The consistent pulmonary changes in animals receiving repeated injections were found to be secondary to a blocking of the veins and capillaries by subendothelial collections of large mononuclear cells. Identical lesions were produced by heat-killed non-hemolytic (gamma type) streptococci and also by certain strains of hemolytic streptococci. The changes apparently represented a peculiar reaction to certain bacterial constituents and will be reported in a subsequent paper.

A group of 50 adult persons was injected intradermally with 0.1 cc. amounts of Birkhaug's standard test toxin. Of the 35 without histories of rheumatic fever, chorea or persistently recurring "sore throats," 12 (34 per cent) gave positive skin reactions; of the 9 without histories of rheumatic fever or chorea, but with histories of "many sore throats," 2 (22 per cent) were positive; and, of the 6 with histories of rheumatic fever, 2 (33 per cent) were positive. Thirteen children convalescent from acute rheumatic fever and with definite damage to one or more valves of their hearts were tested at the House of the Good Samaritan, Boston, through the kind cooperation of the Medical Staff. Of these, 8 (62 per cent) gave positive skin reactions. Control

TABLE I.

*Summary of Results from Cultures from Patients with Acute Rheumatic Fever and from Normal Individuals.*

| Case No. | Rheumatic fever: Age and sex | Blood cultures: Broth No. 1 | Broth No. 2 | Blood agar plates | Clot No. 1 | Clot No. 2 | Stool culture | Urine culture | Throat culture | Controls: Clinical diagnosis | History: R.F. or chorea | Sore throat | Tonsilectomy | Throat culture |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *yrs.* | | | | | | | | | | | | | |
| 1 | 19 ♂ | C-10 | — | 0 | 0 | 0 | 0 | 0 | + | ? gastric path. | 0 | 0 | 0 | + |
| 2 | 25 ♂ | 0 | — | 0 | 0 | — | 0 | 0 | 0 | Cancer of breast | 0 | 0 | 0 | + |
| 3 | 14 ♂ | 0 | — | 0 | 0 | 0 | — | 0 | 0 | Normal | 0 | + | 0 | + |
| 4 | 41 ♂ | 0 | — | 0 | 0 | 0 | 0 | 0 | + | Normal | 0 | ++ | + | + |
| 5 | 45 ♀ | 0 | — | 0 | 0 | 0 | — | — | 0 | Normal | 0 | ++ | + | + |
| 6 | 27 ♂ | 0 | — | 0 | 0 | 0 | 0 | 0 | + | Diabetes | 0 | + | 0 | 0 |
| 7 | 29 ♀ | 0 | — | 0 | 0 | 0 | — | — | + | Normal | 0 | +++ | + | + |
| 8 | 28 ♀ | 0 | — | 0 | 0 | 0 | — | — | + | Normal | 0 | 0 | 0 | 0 |
| 9 | 12 ♀ | C-34 | 0 | 0 | 0 | — | — | — | + | Normal | 0 | + | + | + |
| 10 | 27 ♂ | 0 | 0 | 0 | 0 | — | — | — | + | Normal | 0 | 0 | 0 | + |
| 11 | 31 ♀ | 0 | 0 | 0 | C-30 | — | — | — | + | Normal | 0 | ++ | + | + |
| 12 | 34 ♀ | 0 | 0 | 0 | 0 | — | — | — | + | Normal | 0 | ++ | + | + |
| 13 | 17 ♂ | 0 | 0 | 0 | C-23 | C-23 | — | — | + | Normal | 0 | + | 0 | + |
| 14 | 25 ♀ | C-36 | 0 | 0 | 0 | — | — | — | + | Normal | 0 | + | + | 0 |
| 15 | 19 ♀ | 0 | 0 | 0 | 0 | — | — | — | + | Normal | 0 | + | 0 | + |
| 16 | 35 ♂ | 0 | 0 | 0 | 0 | — | — | — | + | Normal | 0 | + | + | + |
| 17 | 17 ♂ | C-5 | 0 | 0 | C-12 | — | — | — | + | Normal | 0 | 0 | 0 | + |
| 18 | 23 ♂ | 0 | 0 | 0 | 0 | — | — | — | + | Normal | 0 | + | 0 | + |
| 19 | 20 ♂ | 0 | 0 | 0 | 0 | — | — | — | + | Normal | 0 | 0 | 0 | + |
| 20 | 21 ♂ | C-20 | 0 | 0 | 0 | — | — | — | 0 | Normal | 0 | + | 0 | + |
| 21 | 20 ♀ | 0 | 0 | 0 | 0 | — | — | — | + | Normal | 0 | ++ | + | + |
| 22 | 16 ♀ | 0 | 0 | 0 | 0 | — | — | — | 0 | Normal | 0 | 0 | 0 | + |
| 23 | 39 ♂ | 0 | 0 | 0 | 0 | — | — | — | + | Normal | 0 | +++ | + | + |
| 24 | 16 ♀ | 0 | 0 | 0 | 0 | — | — | — | + | Normal | 0 | 0 | 0 | + |
| 25 | 14 ♂ | 0 | 0 | 0 | 0 | — | — | — | + | Normal | 0 | 0 | 0 | + |

0 = no growth under cultures, otherwise absent.
C = contaminated—numbers represent age of culture in days.
+ = gamma type streptococci under cultures, otherwise present.

tests with heated toxin (100°C. for 2 hours) gave results identical with those of the unheated toxin.

*Cultures.*—The results of the various cultures are given in Table I. The blood from each case of acute rheumatic fever was cultured in various ways and no streptococci of any sort were ever recovered.

Organisms were recovered from the broth cultures in 5 cases, as follows:

*Case 1.*—Staphylococci were found on the 10th day. There was no duplicate culture. The clot cultures and blood agar plates were negative.

*Case 9.*—Gram-positive bacilli were found on the 24th day in 1 of the flasks. The duplicate culture, clot culture and blood agar plates were negative.

*Case 14.*—*Staphylococcus albus* was found on the 26th day in 1 of the flasks. The duplicate culture, clot culture and blood agar plates were negative.

*Case 17.*—Gram-positive bacilli were found on the 5th day in 1 of the flasks. The duplicate culture and blood agar plates were negative, but the clot culture showed Gram-positive bacilli on the 12th day.

*Case 20.*—Staphylococci were found on the 20th day in 1 of the flasks. The duplicate culture, clot culture and blood agar plates were negative.

Organisms were recovered from the clot cultures in 3 cases, as follows:

*Case 11.*—*Staphylococcus albus* was found on the 30th day. The broth cultures and blood agar plates were negative.

*Case 13.*—*Staphylococcus albus* was found on the 23rd day in both cultures. The broth cultures and blood agar plates were negative.

*Case 17.*—As above.

The blood agar plates were consistently negative.

Stool and urine cultures were made in 5 out of the first 6 cases. As no colonies were obtained which were suggestive of non-hemolytic streptococci, they were not made from the other cases.

The findings on the blood agar plates from throat swabs were quite different from the negative blood cultures. In the first 5 cases, organisms were recovered from 2 which could not be distinguished, either morphologically or culturally, from the Small and Birkhaug strains. In 18 of the remaining 20 cases, similar organisms were recovered; and it seems likely that they would have been recovered in a higher percentage of the first 5 cases, if, at that time, the characteristic appearance of the colonies on blood agar had been better known.

Throat cultures were made from an equal number of individuals who gave no history of rheumatic fever, chorea or heart disease. By far the majority were from members of the laboratory staff. Non-hemolytic streptococci of the gamma type were recovered from 22 cases (88 per cent), as compared with 20 cases (80 per cent) in the acute rheumatic fever series.

*Serological Tests.*—A number of rabbits were immunized against certain strains of these non-hemolytic (gamma type) streptococci (Small A and B, Birk. 2, RFT 4 and NT 2–8 and 5–2). All of the stock strains and those obtained from rheumatic fever and normal throats were tested, if possible, against all 6 of these antisera. A number of the strains agglutinated spontaneously in salt solution, and, although various media, such as plain broth and phosphate-buffered broth, were used, satisfactory growth could not be obtained.

Preliminary tests showed the presence of a common antigenic base in all strains, for, in a series of increasing dilutions, practically all organisms tested showed complete agglutination as high at 1:160 with all antisera. As the dilutions were carried higher, however, 2 rather distinct groups became apparent—the one represented by Small A and B and the other by all but 1 (Birk. 36) of the Birkhaug strains. The latter group is apparently identical with the group designated by Hitchcock (5) as "Type I." There were a certain number of strains which did not fall into either of these 2 groups; some failed to show any significant agglutination and others were agglutinated by all antisera, but not by the control serum or salt solution.

In doing the routine agglutination tests on all strains the dilutions of the various antisera were such that "group agglutination" was excluded. The results are shown in Table II.

Each strain has been assigned to 1 of 5 groups—Small, Birkhaug, non-agglutinating, agglutinating or spontaneously agglutinating. This division is, in many instances, far from clear-cut, but serves for the purpose of demonstrating the lack of homogeneity among these gamma type streptococci.

Three antisera were prepared from throat strains isolated during the course of this investigation. One (RFT 4) belongs, clearly, to the Birkhaug group, another (NT 2–8) to the agglutinating group and the other (NT 5–2), questionably, to the Small group. It is most interesting to note that, although the strains belonging to the agglutinating group are agglutinated by practically all of the antisera, the antiserum (NT 2–8) of this group fails to agglutinate any strains, in an appreciable amount, other than those belonging to the group.

A résumé of the different groups recovered from the cultures of the normal and rheumatic fever throats is given in Table III. Birkhaug

TABLE II.

*Serological and Cultural Characteristics of Stock and Isolated Strains of Gamma Type Streptococci.*

| Strain | Agglutination tests | | | | | | | | Agglutinin absorption | | Lactose fermentation | Toxin production | Serological group | Remarks |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Salt solution control | Anti-*B. paralyph.* B control 1:640 | Anti-Small A 1:1280 | Anti-Small B 1:1280 | Anti-Birk. 2 1:1280 | Anti-RF 4, 1:2560 | Anti-NT 2-8, 1:2560 | Anti-NT 5-2, 1:1280 | Anti-Small A | Anti-Birk. 2 | | | | |
| Small A | 0 | 0 | +++ | ++ | 0 | 0 | 0 | + | ++++ | 0 | + | 0 | S | |
| B | 0 | 0 | +++ | +++ | 0 | + | 0 | +++ | ++ | − | + | 0 | S | |
| Birk. 1b | 0 | 0 | 0 | 0 | +++ | ++ | 0 | 0 | − | ++++ | + | ++++ | B | |
| 2 | 0 | 0 | 0 | 0 | +++ | ++ | 0 | 0 | + | ++++ | + | 0 | B | |
| 10a | 0 | 0 | 0 | 0 | ++ | + | 0 | 0 | − | ++++ | + | 0 | B | |
| 17 | 0 | 0 | 0 | 0 | +++ | ++ | + | + | − | ++++ | + | 0 | B | |
| 36 | 0 | 0 | ++++ | ++++ | 0 | +++ | 0 | +++ | ++++ | 0 | + | 0 | S | |
| 84 | 0 | 0 | 0 | 0 | ++++ | +++ | 0 | 0 | − | ++++ | + | 0 | B | |
| 167 | 0 | 0 | 0 | 0 | +++ | ++++ | 0 | 0 | − | ++ | − | 0 | B | |
| RFT 1 | 0 | 0 | 0 | 0 | +++ | ++++ | 0 | 0 | − | + | + | 0 | B | |
| 4 | 0 | 0 | 0 | 0 | ++ | ++++ | 0 | 0 | − | ++ | + | 0 | B | |
| 6 | +++ | − | − | − | − | − | − | − | − | + | + | 0 | SA | |
| 7 | 0 | 0 | 0 | 0 | +++ | ++++ | 0 | 0 | − | + | + | 0 | B | |
| 8-2 | +++ | − | − | − | − | − | − | − | − | − | 0 | 0 | SA | |
| 5 | 0 | 0 | 0 | 0 | +++ | +++ | 0 | 0 | − | +++ | + | + | B | |
| 9-1 | +++ | − | − | − | − | − | − | − | − | − | + | 0 | SA | |
| 13 | +++ | − | − | − | − | − | − | − | − | + | + | 0 | SA | |
| 10-3 | 0 | 0 | + | + | ++++ | +++ | +++ | ++ | − | 0 | + | + | A | |
| 5 | 0 | 0 | 0 | 0 | ++ | ++ | 0 | 0 | − | ++ | + | 0 | B | |

| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 11-6 | 0 | 0 | 0 | 0 | +++ | ++++ | 0 | 0 | − | 0 | + | 0 | B | |
| 8 | 0 | 0 | 0 | 0 | ++++ | ++++ | 0 | 0 | − | ++++ | + | 0 | B | |
| 12-3 | 0 | 0 | 0 | 0 | ++++ | ++ | 0 | 0 | − | 0 | + | 0 | B | |
| 7 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | − | − | + | + | NA | |
| 13-1 | 0 | 0 | 0 | 0 | +++ | +++ | 0 | 0 | − | + | + | 0 | B | |
| 14-7 | − | − | − | − | − | − | − | − | − | − | − | + | − | 1 |
| 15-6 | 0 | 0 | 0 | 0 | +++ | ++ | 0 | + | − | 0 | 0 | 0 | B | |
| 7 | 0 | 0 | 0 | 0 | +++ | 0 | 0 | 0 | − | 0 | + | ++ | B | |
| 16-8 | +++ | − | − | − | − | − | − | − | − | − | 0 | 0 | SA | |
| 17-1 | +++ | − | − | − | − | − | − | − | − | 0 | + | 0 | SA | |
| 18-2 | +++ | − | − | − | − | − | − | − | − | − | + | 0 | SA | |
| 3 | 0 | 0 | 0 | 0 | ++++ | ++++ | 0 | 0 | − | 0 | + | 0 | B | |
| 19-1 | 0 | 0 | 0 | 0 | ++++ | ++++ | 0 | 0 | − | 0 | + | − | B | |
| 3 | 0 | 0 | 0 | 0 | ++++ | +++ | ++ | + | − | 0 | 0 | +++ | B | |
| 21-3 | ++++ | − | − | − | − | − | − | − | − | − | + | 0 | SA | |
| 23-1 | 0 | 0 | 0 | 0 | ++++ | ++++ | 0 | + | − | ++++ | + | 0 | B | |
| 24-1 | 0 | 0 | ++++ | ++ | 0 | 0 | ++ | ++ | − | − | 0 | 0 | S | |
| 4 | 0 | 0 | 0 | 0 | +++ | +++ | + | + | − | ++++ | + | ++ | B | |
| 5 | +++ | − | − | − | − | − | − | − | − | − | + | 0 | SA | |
| 6 | 0 | 0 | 0 | 0 | +++ | ++++ | ++ | + | − | +++ | 0 | 0 | B | |
| 25-2 | 0 | 0 | 0 | + | +++ | ++ | + | + | − | +++ | + | 0 | B | |
| 6 | +++ | − | − | − | − | − | − | − | − | − | 0 | 0 | SA | |
| 7 | 0 | 0 | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | − | − | + | 0 | A | 2 |

Agglutinin adsorption: ++++ = complete, +++ = marked, ++ = moderate, and + = slight.

Serological groups: S = Small, B = Birkhaug, A = agglutinating, SA = spontaneously agglutinating, and NA = non-agglutinating.

Remarks: 1 = growth unsatisfactory for agglutination, 2 = raffinose not fermented, and 3 = grown in phosphate-buffered broth for agglutination tests.

TABLE II—*Concluded.*

| Strain | Agglutination tests | | | | | | | | Agglutinin absorption | | Lactose fermentation | Toxin production | Serological group | Remarks |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Salt solution control | Anti-*B. paratyph.* B control 1:640 | Anti-Small A 1:1280 | Anti-Small B 1:1280 | Anti-Birk. 2 1:1280 | Anti-RF 4, 1:2560 | Anti-NT 2-8, 1:2560 | Anti-NT 5-2, 1:1280 | Anti-Small A | Anti-Birk. 2 | | | | |
| NT 1-1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | − | + | + | 0 | NA | 3 |
| 2-6 | 0 | 0 | 0 | 0 | +++ | +++ | 0 | 0 | + | + | + | 0 | B | |
| 8 | 0 | 0 | ++++ | ++++ | 0 | ++ | ++++ | ++++ | +++ | 0 | 0 | 0 | A | |
| 3-1 | 0 | 0 | ++ | ++ | + | 0 | + | ++++ | +++ | − | 0 | 0 | S | |
| 3 | 0 | 0 | 0 | 0 | ++ | +++ | + | 0 | − | + | + | 0 | B | |
| 4-4 | 0 | 0 | 0 | 0 | +++ | +++ | 0 | 0 | − | ++ | + | 0 | B | |
| 5-1 | 0 | 0 | +++ | ++ | ++ | 0 | +++ | +++ | − | + | 0 | 0 | A | |
| 2 | 0 | 0 | ++ | 0 | 0 | 0 | 0 | +++ | ++++ | − | + | 0 | S | |
| 7-2 | 0 | 0 | 0 | 0 | ++ | ++ | 0 | 0 | − | + | + | 0 | B | |
| 4 | 0 | 0 | +++ | + | + | + | + | +++ | − | − | 0 | 0 | S | |
| 9-2 | 0 | 0 | ++ | ++ | + | ++ | + | ++ | − | + | 0 | + | A | |
| 3 | +++ | − | − | − | − | − | − | − | − | − | + | 0 | SA | |
| 10-1 | 0 | 0 | 0 | 0 | +++ | +++ | 0 | 0 | − | +++ | + | 0 | B | |
| 3 | +++ | − | − | − | − | − | − | − | − | − | 0 | 0 | SA | |
| 11-2 | 0 | 0 | 0 | 0 | +++ | ++ | 0 | 0 | − | ++ | + | + | B | |
| 12-6 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | − | − | + | 0 | NA | |
| 13-3 | 0 | 0 | 0 | 0 | ++ | +++ | + | 0 | − | + | + | 0 | B | |
| 15-4 | 0 | 0 | 0 | ++++ | 0 | + | 0 | + | − | − | 0 | 0 | S | |
| 7 | 0 | 0 | 0 | 0 | ++ | ++ | 0 | 0 | − | +++ | + | 0 | B | |
| 16 | 0 | 0 | 0 | 0 | ++ | +++ | + | 0 | − | ++ | + | ++ | B | |

| | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 17-4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | B |
| 6 | 0 | 0 | + | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | PS |
| 18-9 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | B |
| 19-2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | B |
| 20-1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | B |
| 6 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | S |
| 21-2 | + | − | − | − | − | + | + | + | + | + | + | + | + | + | + | + | + | SA |
| 3 | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | S |
| 22-1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | B |
| 6 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | B |
| 8 | + | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | SA |
| 23-5 | + | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | SA |
| 6 | + | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | SA |
| 24-1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | B |
| 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | + | B |
| 25-3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | S |
| 4 | + | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | SA |
| 5 | + | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | SA |
| | | | | | | | | | | | | | | | | | | 3 |

strains (Hitchcock (5)—Type I) were recovered in approximately equal numbers in each of the 2 groups. Many more Small strains were obtained from the normal throats, whereas the other groups occurred with about the same frequency.

Agglutinin absorption tests were carried out with a number of the strains. The results are included in Table II. Many strains that were agglutinated in as high a dilution by the antiserum as the homologous strain failed to show even the slightest degree of absorption of agglutinin. This was particularly true of the strains belonging to the Birkhaug group—for example, from the same throat 1 strain (RFT

TABLE III.

*Summary of the Occurrence of the Various Kinds of Gamma Type Streptococci in Throats of Patients with Acute Rheumatic Fever and of Normal Individuals.*

| Variety of gamma type streptococci | Rheumatic fever throats | | | | Normal throats | | | |
|---|---|---|---|---|---|---|---|---|
| | Occurrence | | Percentage occurrence | | Occurrence | | Percentage occurrence | |
| | All cases | Alone | All cases | Alone | All cases | Alone | All cases | Alone |
| Birkhaug | 14 | 8 | 56 | 32 | 15 | 7 | 60 | 28 |
| Small | 1 | 0 | 4 | — | 8 | — | 32 | — |
| Non-agglutinating | 1 | 0 | 4 | — | 2 | 2 | 8 | 8 |
| Agglutinating | 2 | 0 | 8 | — | 3 | — | 12 | — |
| Spontaneously agglutinating | 9 | 5 | 36 | 20 | 6 | 1 | 24 | 4 |
| None | 5 | — | 20 | — | 3 | — | 12 | — |

11–8) completely absorbed the Birk. 2 antiserum, while the other (RFT 11–6) failed to show any absorption.

All but 1 of the rheumatic fever patients' sera and a number of sera from individuals who gave no history of rheumatic fever were tested for agglutinins against representative strains of the Small and Birkhaug groups. The former sera were also tested, whenever possible, against their own throat strains. Agglutination was considered significant only in the presence of a 3 plus agglutination in a final serum dilution of 1:40, or a 2 plus in this dilution, which was repeated in the 1:80 dilution. In neither the rheumatic fever nor the control sera could agglutinins be demonstrated for Small A or Birk. 2. Five

(Cases 1, 4, 5, 7 and 18) out of 22 rheumatic fever sera and 1 (Case 13) out of 14 control sera agglutinated RFT 4. Three (Cases 9, 11 and 18) out of 17 rheumatic fever sera and 2 (Cases 1 and 3) out of 17 control sera showed agglutinins for RFT 8–5; and 1 (Case 11) out of 17 rheumatic fever sera and 1 (Case 8) out of 17 control sera showed agglutinins for NT 16. Four (Cases 4, 11, 12 and 25) out of 15 rheumatic fever sera agglutinated 1 of the autogenous strains. The only difference noted between the two series was the greater ability of the rheumatic fever sera to agglutinate RFT 4. As this particular strain was frequently spontaneously agglutinated and, hence, gave proof of instability, too much significance should not be attached to the observation.

*Toxin Production.*—All strains isolated from the rheumatic fever and normal throats were tested for the production of a soluble skin-reacting toxin. The tests were carried out in the skin of an individual known to react strongly to Birkhaug's standard test toxin. The results of these skin tests are recorded in Table II. Of the rheumatic fever strains, 7 gave positive skin reactions, but only 3 were of sufficient intensity (erythema 1.0 cm. or more in diameter) to be significant in a dilution of the filtrate as low as 1:10. Of the 40 normal throat strains, 4 were positive, 2 being of moderate intensity.

In order to make certain of the identity of these skin reactions with those of Birkhaug, 37 adult individuals were injected, intradermally and at the same time, with 1 (Birk. 1b, 1:10) of these toxins and with Birkhaug's standard toxin. In 33 instances the skin reactions to both toxins were identical (9 positive and 24 negative), whereas in 4 they disagreed (Birk. 1b positive with standard toxin negative in 3 and standard toxin positive with Birk. 1b negative in 1).

## DISCUSSION.

The comparison of the Small and Birkhaug strains of non-hemolytic streptococci would seem to indicate that, although they are very closely related, they are not identical. This finding agrees with the observations of Hitchcock (5).

There are two outstanding discrepancies when the experimental findings reported herein are compared with those of Small and Birkhaug. In the first place, no joint changes, such as those described by

Small (2), were ever observed in the rabbits which had received repeated large doses of living organisms. It is true that fresh vegetations on the valves and papillary muscles of the heart were observed on 2 occasions, but this might be expected following the intravenous injection of almost any living organism in such large numbers. Furthermore, it should be borne in mind that rabbits with a streptococcus septicemia, regardless of the source or variety of the streptococci, frequently show acute joint involvement along with other localized foci of infection. Secondly, it has been impossible to demonstrate that the majority of these strains of streptococci produce a soluble skin-reacting toxin. Birkhaug (3) states that 72 per cent of his non-methemoglobin-producing streptococci gave strongly positive skin reactions in dilutions as high as 1:100. Such a toxin was found in only 1 (Birk. 1b) of Birkhaug's 7 strains, and dilutions as low as 1:10 of the other 6 failed to give definite skin reactions. Although no phenol was added before filtration and although the filtrates were tested oftentimes within a few days after filtration, rather than after 1 month in the ice box, as Birkhaug did, it is difficult to conceive of either procedure as being capable of influencing the final result. As previously stated, the 1 toxic filtrate (Birk. 1b) gave practically identical skin reactions as the Birkhaug standard test toxin, when the tests were carried out simultaneously.

Non-hemolytic streptococci of the gamma type were never recovered in the blood cultures from 25 cases of acute rheumatic fever. Bacteria were occasionally isolated, but they were never found consistently in all cultures. Those recovered were of the type to be expected as a result of contamination, and they were usually found only after 10 or more days incubation. From the papers of Small (2) and Birkhaug (3) it would appear that the former obtained his organisms in 2 out of 31 cases and the latter in 2 out of 36. Hitchcock (5) also obtained 2 strains from blood cultures of patients with rheumatic fever. It must be acknowledged that the present series of 25 cases is small and different results might obtain in a larger series. The blood cultures also failed to reveal the presence of streptococci of the alpha type, such as those described by Clawson (1).

From the throats of 20 out of 25 cases of acute rheumatic fever non-hemolytic streptococci were recovered which were identical,

morphologically and culturally, with the Small and Birkhaug strains. As judged by agglutination, the majority of strains were similar to those of the latter. As has been previously mentioned, the Birkhaug strains apparently correspond to the Type I of Hitchcock (5). Agglutinin absorption tests with these Birkhaug similar strains would seem to indicate that 3 out of the 18 strains were identical with Birk. 2. There were many strains, however, which, although they were agglutinated by anti-Birk. 2 serum in high dilution, showed a complete absence of ability to absorb the specific agglutinin from the same serum. Reciprocal agglutinin absorption tests were not carried out. When tested for toxin production, only 3 filtrates from the 34 strains were found which were able to bring about a definite skin reaction in a dilution as low as 1:10.

Similar non-hemolytic streptococci were recovered from 22 out of 25 normal throats. The only difference noted in this series, as compared with the strains isolated from rheumatic fever throats, was a considerably higher number of Small strains. The rather universal presence of these streptococci in both the rheumatic and non-rheumatic throats and the apparent predominance of Birkhaug similar strains, as determined by agglutination, agree very closely with the figures of Hitchcock (4).

From a comparison of these 2 series it would seem reasonable to conclude that non-hemolytic (gamma type) streptococci are of relatively common occurrence in the human throat. The fact that practically all strains are agglutinated by relatively low dilutions of antisera of various type strains argues for a common antigenic base. When the dilutions are carried higher, however, it becomes apparent that the strains are far from identical. Agglutinin absorption tests bring the heterogeneity of this group even more in evidence.

Sera from rheumatic fever patients have failed to show agglutinins for the Small or Birkhaug strains. It must be acknowledged that these sera were obtained during the acute stage of the disease and that such agglutinins might appear subsequent to improvement or recovery.

From the work reported here, very little can be said concerning the etiologic importance of these streptococci in relation to the conception of acute rheumatic fever as a manifestation of bacterial allergy

(Zinsser (6, 7), Swift, Derick and Hitchcock (8) and Birkhaug (9)). The findings, however, fail to indicate that such streptococci should be considered as the specific etiologic factor in acute rheumatic fever. Kaiser (10) has reported a large series of cases which have been tested for skin reactivity to a soluble toxin obtained from 1 of these streptococci and there can be no doubt but that children with a history of rheumatic fever or chorea give a higher percentage of positive reactions than children without such a history. There are, however, at least two facts which should be considered. In the first place, this toxin is extremely thermostabile, a fact which distinguishes it sharply from the true exotoxins, such as diphtheria and tetanus toxins, and makes it hardly comparable, even, to Dick toxin, which is relatively thermostabile. Secondly, very little is known concerning skin reactivity, in general, to non-specific foreign proteins during or following different diseases. The recent work of Irvine-Jones (11) brings out the fact that persons suffering from rheumatic fever, particularly during the acute stage, are relatively skin-sensitive to the filtrates from various sorts of streptococcic cultures. Whether or not such individuals are sensitive to the filtrates from cultures of other types of bacteria or to protein substances of primarily non-toxic nature remains to be demonstrated. Kaiser's observations certainly point to a specific relationship, but until more is known concerning the nature of such toxins and concerning the significance of skin reactivity in various diseases it hardly seems justifiable to draw any definite conclusions.

## SUMMARY AND CONCLUSIONS.

1. Blood cultures from 25 cases of acute rheumatic fever were negative for non-hemolytic streptococci of both the alpha and gamma types.

2. Non-hemolytic (gamma type) streptococci were frequently recovered from the throats of patients with this disease.

3. Similar organisms were recovered just as frequently from the throats of normal individuals.

4. Although these non-hemolytic streptococci were morphologically and culturally identical, not only amongst themselves, but also when compared with stock Small and Birkhaug strains, all, including the latter, have failed to show any noteworthy degree of homogeneity.

5. Representative strains of these streptococci have proved to be relatively non-pathogenic for rabbits following intravenous injection.

6. These organisms, with a few exceptions, have failed to produce soluble skin-reacting toxins comparable to Birkhaug's standard test toxin.

7. The foregoing facts seem to invalidate the assumption that any of these non-hemolytic streptococci play a specific rôle in the etiology of acute rheumatic fever.

We wish to express our indebtedness to Miss Miriam MacKay for technical assistance.

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# THE INITIATION OF GROWTH OF CERTAIN FACULTATIVE ANAEROBES AS RELATED TO OXIDATION-REDUCTION PROCESSES IN THE MEDIUM.

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The expression "value of a bacteriological medium" is a loose one; it probably covers a number of characteristics of that medium, many of which are perhaps unrelated. The following are examples of the points of view from which the medium may be considered in this relation.

1. Initiation of growth: (*a*) size of minimum inoculum required for growth to develop, (*b*) duration of the lag period.

2. Density of growth: number of cells developing per unit capacity of medium.

3. Viability of the cultures.

This paper will be limited to a survey of some of the conditions which affect the size of the inoculum required to intiate growth.

## HISTORICAL.

It is a well known fact that the growth of organisms difficultly cultivable is rendered much easier—even in unfavorable media—by the use of large inocula. This phemonenon is probably related to what has been termed "allelocatalysis" by Robertson (1) in his studies on transplants of protozoa, and "communal activity," by Churchman and Kahn (2) in their investigations on the bacteriostatic action of certain dyes.

The work of Valley and Rettger (3) indicates that many organisms grow slowly or not at all until a certain minimum concentration of carbon dioxide is present in the medium; the suggestion has been made that the introduction of a large inoculum may hasten the production of a favorable concentration of $CO_2$.

Gillespie (4) observed that "it requires much smaller numbers of pneumococci to start growth on agar than are required to start a growth in broth." He found also that the same result as with agar could be obtained by seeding the pneumococci on a fragment of filter paper on the surface of the broth. According to him, "this phenomenon is probably dependent entirely on physical differences

in the two kinds of media, and bears some relation to the differences in possibilities for diffusion in the two media."

The possible relation of minimum inoculum to oxidation-reduction processes has been expressed several times in the literature. In the course of his studies on the physiology of *B. lepisepticum*, Webster (5) observed that an inoculum of at least 100,000 cells (per 5 cc. broth), was necessary for growth to develop under aerobic conditions. On the other hand, growth occurred with an inoculum of only a very few cells when the culture was incubated under anaerobic conditions, or in the presence of sterile blood.

Burnet (6) showed that nutrient agar plates, exposed to light, while still capable of growing staphylococcus when heavily seeded, did not allow growth of "isolated" organisms. ("Isolated" organisms were obtained by spreading over the plate a very dilute suspension of bacteria.) But such "isolated" organisms would grow in the neighborhood of colonies of the same or other organisms, obtained by local heavy seedings. Nutrient agar which had been exposed to light and then heated for 30 minutes at 100°C. gave normal growth, unless exposure to light had been extremely prolonged. The explanation offered was that exposure to light resulted in the formation of a peroxide-like substance with a bacteriostatic action; reducing substances, formed in the course of metabolism in the large colonies (obtained by local heavy seedings) diffused through the agar and reduced the peroxides, thus allowing growth of "isolated" organisms around the colonies. Destruction of the peroxide was also obtained by heating the medium. Working with an anaerobe, *B. sporogenes*, Quastel and Stephenson (7) observed that this organism could be grown under apparent "aerobic" conditions, in tryptic digested broth, by the use of a large inoculum. A very small inoculum was sufficient to initiate "aerobic growth" when 0.1 per cent cysteine was added to the broth. Reduced glutathione and thioglycollic acid were found to play the same rôle as cysteine. According to Quastel and Stephenson, the mechanism of the process is as follows: In the course of its metabolism, *B. sporogenes* produces reduced compounds giving the −SH reaction; the object of a large inoculum is to introduce enough of these reduced sulphydryl compounds to provide reducing conditions in the broth. The addition of cysteine serves of course the same purpose.

W. M. Clark (8) had previously pointed out that actively growing anaerobic cells, if not overtaxed, can establish their own reducing conditions even in the presence of a certain amount of molecular oxygen.

Aubel and Aubertin (9) seeded different organisms into tubes of glucose agar and observed at what levels of the agar growth developed. By observing the reduction of indicators of oxidation-reduction potentials in sterile tubes of the same medium, and comparing the findings with the results of the growth experiments, they concluded that the life of strict anaerobes is possible only when the rH of the medium is lower than 12.

Before attempting to analyze the mechanism whereby the oxidation-reduction properties of the medium control the minimum inoculum required to initiate growth, it is important to realize that bacterial cells themselves possess active and independent oxidation-reduction systems. The manifestations and nature of these systems are especially well known in the case of Pneumococcus.

Avery and Neill (10) have described a number of different oxidation-reduction processes which are exhibited by cultures or sterile extracts of pneumococci; such are the consumption of molecular oxygen, the production of peroxide, the oxidation of hemotoxin, the oxidation of hemoglobin to methemoglobin, and the oxidative destruction of various endocellular enzymes. The same cultures or extracts of pneumococci also reduce methylene blue to methylene white and methemoglobin to hemoglobin when the system is kept under anaerobic conditions.

These active oxidation-reduction systems of the cells consist of two components: (1) a cellular thermolabile constituent which is not removed by washing, (2) thermostable autoxidizable substances which are lacking in washed cells and which are not necessarily of pneumococcus origin, since they may be supplied by muscle infusion and yeast extract.

It is probable that a complete understanding of the influence of oxidation-reduction processes in bacterial growth will require a study of the interrelations between the oxidation-reduction systems of the media and of the cells.

## EXPERIMENTAL.

The influence of the nature of the medium and of environmental conditions on the size of the minimum inoculum giving rise to growth, was studied by the following technique.

Tubes containing 5 cc. of the medium under consideration were inoculated with varying amount of culture. These inocula are referred to as $10^{-1}$, $10^{-2}$, $10^{-3}$, $10^{-4}$, $\ldots.10^{-8}$, corresponding to 0.1....0.00000001 cc. of a 12 hour culture in plain broth.

The cultures used were: Pneumococcus S forms, Type I (1/200/5), II (D/39/40), III (A/66/64), and R forms derived from Types I (1/193/R), II (D/39/R), and III (M/3/R). Human strains of hemolytic streptococcus, L, S/43, S/23/Glossy, S/23/45 Matt. *Staphylococcus aureus.*

Inocula were never taken from blood cultures, but from the third transfer in plain broth in order to prevent the carrying over of some of the blood constituents into the medium to be tested.

These plain broth cultures were plated on blood agar to determine the number of organisms present in the inoculum. There was of course some variation, but in general, plates inoculated with $10^{-7}$ of plain broth cultures of Pneumococcus or human strains of *Streptococcus hæmolyticus* showed only a few colonies (1 to 10). With Staphylococcus, the last positive plates were obtained with $10^{-9}$ cc. inoculum.

The plain broth was prepared from meat infusion according to the standard method. Fairchild's peptone (lot 280630) was used in the preparation. Cysteine solutions were prepared from cysteine hydrochloride and autoclaved. As the neutralized solution oxidizes very rapidly, while the acid solution is more stable, the latter was neutralized with sterile NaOH only at the time of being used. "Anaerobic" conditions were provided by sealing the cultures with a 2 cm. layer of sterile vaseline.

TABLE I.

*Growth of Pneumococcus and Streptococcus in the Presence of Different Concentrations of Blood.*

| Medium | Smallest inocula with which growth was obtained | | | |
|---|---|---|---|---|
| | Pneumococcus II | | *S. hæmolyticus* (human strains) | |
| | "S" D/39/40 | "R" D/39/R | L | S/43 |
| Plain broth | $10^{-2}$ | $10^{-2}$ | $10^{-2}$ | $10^{-3}$ |
| Plain broth + 0.001 per cent blood | $10^{-2}$ | $10^{-2}$ | $10^{-2}$ | $10^{-2}$ |
| Plain broth + 0.01 per cent blood | $10^{-4}$ | $10^{-4}$ | $10^{-5}$ | $10^{-4}$ |
| Plain broth + 0.1 per cent blood | $10^{-8}$ | $10^{-8}$ | $10^{-8}$ | $10^{-7}$ |
| Plain broth + 1 per cent blood | $10^{-7}$ | $10^{-6}$ | $10^{-6}$ | $10^{-7}$ |

### *I. Growth of Streptococcus and Pneumococcus in Blood Broth.*

Blood broth is known to be an ideal medium for the growth of pneumococci and streptococci. The attempt was made to ascertain what concentration of blood is necessary to insure growth with the smallest possible inocula.

*Experiment 1.*—The plain broth used had been prepared 1 week before the test. Sterile rabbit blood was added to the medium in amounts sufficient to give final concentrations varying from 1 per cent to 0.001 per cent. Table I indicates, in cubic centimeters of plain broth cultures, the smallest inocula with which growth was obtained.

According to this experiment, blood is still active in a dilution of 0.01 per cent. It may be doubted whether the action of such a small amount

corresponds to the addition of some nutrient lacking in the broth. There have been suggestions that the action of the blood is due to the fact that it contains a peroxidase or a catalase and perhaps also the V factor which has been associated with yeast extract. It may be recalled here that Thjotta and Avery (11) found that two factors (X and V) are essential for the growth of certain strains of influenza bacillus. The factor V appears to be of the nature of a vitamine and can be supplied in the form of yeast extract; X was claimed to be an iron compound and hematin was used as its source in routine technique. X exhibits peroxidase and catalase activity. Both X and V are present in the blood.

Attempts have been made therefore to simulate the action of blood by the addition of certain iron compounds (possessing catalase, peroxidase, and oxidase activity) in the presence or in the absence of yeast extract.

### *II. The Influence of Iron Compounds on the Growth of Pneumococcus.*

*Experiment 2.*—In these tests, a large number of iron compounds were used.[1] Among them may be mentioned.

Active $Fe_2O_3$ (Siderac)<br>Active $Fe_2O_3$ (Baudisch) } which exhibit both peroxidase and catalase activity.

Inactive $Fe_2O_3$ (Siderac)<br>Inactive $Fe_2O_3$ (Baudisch) } which exhibit only catalase activity.

Sodium pentacyano aquo-ferro salt<br>Sodium pentacyano aquo-ferri salt<br>Sodium pentacyano amino-ferri salt } which exhibit both catalase and peroxidase activity.

In order to obtain comparative results, the following technique was used. The oxides were added to test-tubes in amounts of 100 to 500 mg. and autoclaved. 5 cc. of sterile broth were added later under sterile conditions. As to the ferro-aquo, ferri-aquo, and ferri-amino salts which are soluble and heat-labile, their solutions were filtered through Berkefeld filters and the filtrates added under aseptic conditions to sterile broth, the final concentrations varying from 1/1,000 to 1/1,000,000.

The tubes were inoculated with 8 hour cultures (in plain broth) of the different types of Pneumococcus. The results may be summarized as follows:

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[1] All the compounds were obtained through the courtesy of Dr. Baudisch.

It is apparent that in no case was the plain broth improved by the addition of iron compounds. On the contrary, some of them seem to have a toxic action; such are the Siderac oxides and the ferri-amino salt.

The results were not changed when yeast extract was added to these media.

It is apparent that these iron compounds with or without the addition of yeast extract, do not give to plain broth those growth-promoting properties which are supplied by blood.

TABLE II.

*Influence of Iron Compounds on the Growth of Pneumococcus (II R).*

| Medium | Inoculum (in cc.) | | | | | |
|---|---|---|---|---|---|---|
| | $10^{-1}$ | $10^{-2}$ | $10^{-3}$ | $10^{-4}$ | $10^{-5}$ | $10^{-6}$ |
| Plain broth | +* | + | −* | − | − | − |
| Blood broth | + | + | + | + | + | + |
| Plain broth + active $Fe_2O_3$ (Siderac) | − | − | − | − | − | − |
| Plain broth + active $Fe_2O_3$ (Baudisch) | + | + | − | − | − | − |
| Plain broth + inactive $Fe_2O_3$ (Siderac) | − | − | − | − | − | − |
| Plain broth + inactive $Fe_2O_3$ (Baudisch) | + | + | − | − | − | − |
| Plain broth + ferro-aquo salt | + | + | − | − | − | − |
| Plain broth + ferri-aquo salt | + | + | − | − | − | − |
| Plain broth + ferro-amino salt | + | − | − | − | − | − |

* In the presentation of all these results, + or − indicates that growth did or did not develop.

## *III. Variations of the Growth-Promoting Properties of Plain Broth with Aging of This Medium.*

In the course of these investigations, it has been repeatedly observed that when plain broth has been recently autoclaved, it allows the growth of very minute inocula of Pneumococcus. In order to establish this phenomenon more definitely, the following experiment was carried out.

*Experiment 3.*—Tubes containing 5 cc. of plain broth (1 week old), were autoclaved for 15 minutes, cooled down, and immediately inoculated with varying amounts of pneumococcus culture (D/39/R). Other tubes of the same autoclaved broth were kept for different intervals of time before being inoculated. Table III shows the highest dilution of inoculum with which growth was obtained at the different periods.

Similar results were obtained with other strains of Pneumococcus (Types I, II, III, virulent and avirulent) and with human strains of hemolytic streptococcus. Boiling the broth for 1 hour had the same effect as autoclaving.

With Staphylococcus, growth in "unboiled" broth (3 weeks old) was obtained up to $10^{-5}$ cc. inoculum, while it took place up to $10^{-8}$ in recently autoclaved broth.

TABLE III.

*Growth of Pneumococcus (D/39/R) in Broth Inoculated at Different Times after Autoclaving.*

| Medium | Inoculum (in cc.) | | | | | | |
|---|---|---|---|---|---|---|---|
| | $10^{-1}$ | $10^{-2}$ | $10^{-3}$ | $10^{-4}$ | $10^{-5}$ | $10^{-6}$ | $10^{-7}$ |
| "Unautoclaved" broth | + | + | − | − | − | − | − |
| Broth inoculated immediately after autoclaving | + | + | + | + | + | + | + |
| Broth inoculated 2 hrs. after autoclaving | + | + | + | + | + | + | + |
| Broth inoculated 4 hrs. after autoclaving | + | + | + | + | + | − | − |
| Broth inoculated 8 hrs. after autoclaving | + | + | + | + | − | − | − |
| Broth inoculated 12 hrs. after autoclaving | + | + | + | − | − | − | − |
| Broth inoculated 24 hrs. after autoclaving | + | + | − | − | − | − | − |
| Broth inoculated 48 hrs. after autoclaving | + | + | − | − | − | − | − |
| Broth inoculated 1 week after autoclaving | + | + | − | − | − | − | − |
| Broth inoculated 3 weeks after autoclaving | + | − | − | − | − | − | − |

It is very important to point out that the effects of boiling or autoclaving are not limited to a short time after the treatment, but extend over 12 hours at least. As has been pointed out elsewhere (12) this indicates that the effect of the treatment is not limited to a mechanical removal of oxygen.

It has already been suggested that the effect of autoclaving or boiling is due to a breaking down or a reduction of oxidized substances

formed by the autoxidation of some constitutents of the broth. If this is the case, similar results should be obtained by chemical methods of reduction. Experiments 4, 5, and 6 are examples of such methods.

*Experiment 4.*—A lot of broth (2 weeks old) was divided into 2 portions, 1 of which was reduced by hydrogen in the presence of palladinized asbestos, the asbestos being later filtered out in a nitrogen atmosphere. The untreated broth was used as control.

TABLE IV.

*Growth of Pneumococcus (D/39/R) in Broth Reduced by Hydrogen.*

| Medium | Inoculum (in cc.) | | | | | | |
|---|---|---|---|---|---|---|---|
| | $10^{-1}$ | $10^{-2}$ | $10^{-3}$ | $10^{-4}$ | $10^{-5}$ | $10^{-6}$ | $10^{-7}$ |
| Plain broth | + | + | − | − | − | − | − |
| Reduced broth | + | + | + | + | + | + | + |

TABLE V.

*Influence of Vaseline Seal on the Growth of Different Organisms in Plain Broth.*

| Organism | Smallest inocula with which growth developed | |
|---|---|---|
| | Aerobic cultures | Sealed cultures |
| Pneumococcus D/39/R | $10^{-2}$ | $10^{-4}$ |
| A/66/64 | $10^{-1}$ | $10^{-4}$ |
| *S. hæmolyticus* (L) | $10^{-2}$ | $10^{-4}$ |
| S/43 | $10^{-1}$ | $10^{-3}$ |
| *Staphylococcus aureus* | $10^{-5}$ | $10^{-8}$ |

The 2 portions were transferred to test-tubes (5 cc. per tube), and their growth-promoting power compared (see Table IV).

It appears that reducing the broth by hydrogen in the presence of palladinized asbestos affects its value in the same manner as boiling or autoclaving.

It has been indicated elsewhere (12) that plain broth kept under vaseline seal develops a reduction potential corresponding to reduced indigo disulfonate. In view of the results of Experiment 4, it was interesting to test whether incubation under vaseline seal would affect the size of the inoculum required to initiate growth.

*Experiment 5.*—Test-tubes containing 5 cc. of broth (2 weeks old) were inoculated with Pneumococcus, human strains of hemolytic streptococcus, and *Staphylococcus aureus;* some of the tubes were sealed with vaseline immediately after inoculation, while the others were incubated under aerobic conditions. Table V indicates the smallest inocula with which growth developed.

This experiment, the results of which have been repeatedly confirmed, show that initiation of growth is facilitated under vaseline seal. However, the results obtained were not so striking as the ones obtained with "boiled" or "autoclaved" broth, or, as shown later, by the addition of cysteine or ascitic fluid to the broth.

Sterile blood was added to the tubes which had failed to grow, but no growth developed on further incubation, thus indicating that the inoculum was dead. In the case of Pneumococcus, autolysis of the cells may account for the fact, but this explanation does not hold for Streptococcus; the significance of these observations will be discussed later.

### *IV. Growth of Pneumococcus, Streptococcus hæmolyticus, and Staphylococcus in Cysteine Broth.*

Cysteine is an active reducing agent, capable of reducing indigo carmine (13). Its action in facilitating the growth of certain anaerobes has been reported and Quastel and Stephenson (7) suggested that, in the case of *B. sporogenes*, the beneficial action of cysteine was due to the establishment in the medium of a favorable reduction potential.

A preliminary experiment showed that, in the presence of 0.1 per cent cysteine, growth of Pneumococcus (D/39/R), human strains of *S. hæmolyticus* (L), and *Staphylococcus aureus* could be obtained with inocula of $10^{-7}$, $10^{-7}$, and $10^{-9}$ cc. of broth cultures, respectively.

*Experiment 6.*—Plain broth was treated with different concentrations of cysteine (0.002 per cent, 0.005 per cent, 0.01 per cent, 0.02 per cent, 0.03 per cent, 0.1 per cent), in order to determine the smallest concentration at which that substance would be active. The tubes were inoculated 12 hours after treatment and incubated under aerobic conditions for 72 hours. The organisms used were those described in the experimental methods.

This experiment shows that cysteine was still active in as high a dilution as 0.005 per cent. With very small inocula ($10^{-6}$, $10^{-7}$, $10^{-8}$) growth developed only after 2 to 3 days, this indicating that cysteine was still active at the time.

TABLE VI.

*Smallest Inocula (in cc.) with Which Growth Developed in Presence of Different Concentrations of Cysteine.*

| Organism | Concentration of cysteine | | | | | | |
|---|---|---|---|---|---|---|---|
| | 0.1 | 0.03 | 0.02 | 0.01 | 0.005 | 0.002 | 0 |
| | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* | |
| Pneumococcus 1/193/R | $10^{-7}$ | $10^{-8}$ | $10^{-8}$ | $10^{-8}$ | $10^{-7}$ | $10^{-4}$ | $10^{-2}$ |
| 1/200/5 | $10^{-8}$ | $10^{-7}$ | $10^{-7}$ | $10^{-6}$ | $10^{-6}$ | $10^{-3}$ | $10^{-2}$ |
| D/39/R | $10^{-8}$ | $10^{-8}$ | $10^{-8}$ | $10^{-6}$ | $10^{-8}$ | $10^{-5}$ | $10^{-3}$ |
| D/39/45 | $10^{-7}$ | $10^{-7}$ | $10^{-8}$ | $10^{-7}$ | $10^{-8}$ | $10^{-3}$ | $10^{-2}$ |
| M/3/R | $10^{-8}$ | $10^{-8}$ | $10^{-8}$ | $10^{-6}$ | $10^{-7}$ | $10^{-4}$ | $10^{-3}$ |
| A/66/64 | $10^{-6}$ | $10^{-8}$ | $10^{-7}$ | $10^{-6}$ | $10^{-6}$ | $10^{-3}$ | $10^{-2}$ |
| *S. hæmolyticus* S/23/G | $10^{-8}$ | $10^{-7}$ | $10^{-7}$ | $10^{-6}$ | $10^{-6}$ | $10^{-3}$ | $10^{-2}$ |
| S/23/76 | $10^{-7}$ | $10^{-7}$ | $10^{-7}$ | $10^{-7}$ | $10^{-6}$ | $10^{-4}$ | $10^{-2}$ |
| *Staphylococcus aureus* | $10^{-9}$ | $10^{-9}$ | $10^{-8}$ | $10^{-8}$ | $10^{-8}$ | $10^{-7}$ | $10^{-5}$ |

TABLE VII.

*Effect of Fumaric and Succinic Acids (0.2 Per Cent in Weight) on the Growth of D/39/R.*

| Medium | Inoculum (in cc.) | | | |
|---|---|---|---|---|
| | $10^{-1}$ | $10^{-2}$ | $10^{-3}$ | $10^{-4}$ |
| Plain broth | + | + | − | − |
| Plain broth + succinic acid | + | + | − | − |
| Plain broth + fumaric acid | + | + | − | − |

TABLE VIII.

*Smallest Inocula Required for Growth to Develop in Plain Broth and Ascitic Fluid Broth.*

| Organism | Plain broth | Ascitic fluid broth | |
|---|---|---|---|
| | | Heated | Unheated |
| Pneumococcus (D/39/R) | $10^{-1}$ | $10^{-2}$ | $10^{-7}$ |
| *S. hæmolyticus* (L) | $10^{-1}$ | $10^{-2}$ | $10^{-6}$ |
| *Staphylococcus aureus* | $10^{-4}$ | ? | $10^{-9}$ |

It is interesting to test the influence of other reducing organic substances. Succinic acid is such a substance, its oxidized form being fumaric acid.

*Experiment 7.*—The effect of the addition of plain broth (2 weeks old) of fumaric and succinic acids on the aerobic growth of Pneumococcus is recorded in Table VII.

As far as the experiment shows, the addition of succinic and fumaric acids does not affect the growth of Pneumococcus (D/39/R). The only difference observed was that growth developed in 24 hours in the succinic acid tubes inoculated with $10^{-2}$ cc., while it took 48 hours in the plain broth and fumaric acid broth with the same inoculum.

*Experiment 8.*—Ascitic fluid broth (5 per cent ascitic fluid, added to 3 weeks old plain broth) was tested for its value in the initiation of growth of small inocula of facultative anaerobes. In some of the tubes the ascitic fluid had been boiled before addition to the broth. A typical protocol is presented in Table VIII.

These results have been confirmed with many other strains of the same bacterial species. They show that unheated ascitic fluid broth allows the growth of very small inocula of the organisms used. Ascitic fluid seems to be inactivated by boiling.

## DISCUSSION.

It is apparent that the growth-promoting properties of plain broth do not remain constant. Fresh broth used within a few hours after its preparation, usually grows Pneumococcus, human strains of hemolytic streptococcus, and *Staphylococcus aureus*, even when one or a very few cells are used as inoculum ($10^{-7}$, $10^{-9}$ cc. of plain broth cultures). Within 24 hours, the broth has so changed that it can grow the same organisms only if large inocula are used ($10^{-2}$, $10^{-3}$ cc.) and after 3 weeks, the broth has become very poor. However, this same broth can be restored to its original value by autoclaving, boiling, or reducing with hydrogen in the presence of palladinized asbestos, as well as by the addition of small amounts of reduced cysteine, but not by the addition of succinic acid. The addition of ascitic fluid and of very small amounts of blood also serves to restore its value.

It is probably fallacious to attempt to explain the results obtained with all these procedures from one single point of view. However, it may be worth while to point out some possible correlations.

Let us first consider the "deterioration" of plain broth on aging. The fact that this "deterioration" can be completely or partially corrected by reducing the medium with hydrogen and with cysteine, or by keeping it under vaseline seal (condition under which the broth is known to develop highly reducing potentials (12)), suggests strongly that this "deterioration" is connected with some oxidation or oxygenation process. The influence of autoclaving and boiling may lead to the same conclusion.

It is hardly probable that the results can be explained by a purely mechanical removal of the oxygen. Studies with indicators of oxidation-reduction potentials have suggested that boiling or autoclaving has more than this purely mechanical effect (12). It has been shown that recently boiled or autoclaved broth maintains the indophenols in a reduced condition for several hours, even under conditions of active aeration. This indicates that the oxidation potential of the broth is lowered either by the reduction of some reversible system of oxidation-reduction, or by the breaking down of a highly oxidized system. A similar conclusion can be reached from a consideration of the results of Experiment 3, in which it is shown that the influence of boiling lasts for over 12 hours—even if the broth is thoroughly aerated.

Blood is an active reducing agent and is also known to possess catalytic properties. Whether the high dilution at which it is still active justifies the assumption that its action is due to a quantitative reduction is problematical. It is possible that the action of blood results in a catalytic breaking down of the peroxides that appear to form in aerated plain broth. In such a case, the inactivity of the iron catalysts used in Experiment 2 would have to be traced to a lack of specific affinity between the iron compounds and the "organic peroxide" of the broth. Finally, it must be remembered that blood introduces formed elements in the medium. It is certain that a bacterial cell adsorbed by, or simply in the vicinity of, a blood corpuscle, finds local conditions markedly different from those prevailing in the rest of the medium. That such conditions are highly reducing is probable.

Little is known of the properties of ascitic fluid. Confirming others

(14), we have found that its reducing capacity is very small, but the factor intensity should be measured by electrometric methods. On the other hand the sample of ascitic fluid used in these experiments gave the peroxidase and catalase tests; it is possible that these catalytic properties were due to the presence of occult blood in the fluid. In such a case, the action of ascitic fluid would be only another example of the activity of small concentrations of blood.

Concerning the reducing intensity of succinic acid, Thunberg (15) reported that equimolecular mixtures of succinic and fumaric acids kept under anaerobic conditions with methylene blue (in the presence of succinodehydrogenase) give only a partial reduction of the dye. This indicates that the system has a very low reduction intensity, much inferior to that of cysteine (which can reduce indigo carmine) and may account for its inactivity. Furthermore, it is probable that questions of specific affinity have to be considered here.

Any problem of growth is of course a problem of reaction between the organism and the environment. So far, our discussion has been concerned only with the environment. Let us now consider the cell as affected by these environmental conditions. We know that the pneumococcus cell is extremely sensitive to changes in the oxygen tension, its thermostable autoxidizable substance becoming alternately oxidized or reduced according to whether the conditions are aerobic or anaerobic, and the thermolabile cellular constituent becoming irreversibly inactivated by slow oxidation. It is to be expected, therefore, that the condition of the cell will be greatly affected by the oxidation potential of the medium. It is also probable that the condition of oxidation or reduction of the cell is not without effect on its ability to multiply.

Our results seem to be best explained by the following hypothesis.

Oxidation processes bear a definite relation to the size of inoculum required to initiate the growth of Pneumococcus, Streptococcus, and Staphylococcus. As we prepare it, plain broth contains reducing autoxidizable substances. In contact with air these substances give rise either to the oxidized form of a reversible oxidation-reduction system, or, by irreversible oxidation, to highly oxidized substances (of the nature of so called "peroxides"). The organisms studied in this work require a medium with a definite range of oxidation-reduction potential for cell multiplication to occur (7, 9, 16). "Oxidized"

broth has a potential outside this range. Such a condition may be corrected in different ways.

(*a*) The bacterial cell itself is equipped with an active reducing system. When a large inoculum is used, enough reducing substances are introduced (from the bacterial cells and the reduced medium carried with them) to reduce the "oxidized" broth and bring it back to favorable conditions.

(*b*) Addition of cysteine and blood, and reduction by means of hydrogen, serve the same purpose. Blood may be active also by catalyzing the breaking down of the hypothetical "peroxides."

(*c*) It is more difficult to see how boiling or autoclaving may bring about the reduction of a reversible system; it is more probable that they cause a breaking down of the oxidized substance.

(*d*) When incubated under vaseline seal, plain broth develops a high reduction potential (12). It may seem surprising that, under such conditions, the results are not so favorable as those obtained by reducing the broth in some other way. A possible explanation is that the reduction of the "oxidized" substances of the broth under vaseline seal is only a slow process; before they are all reduced by the broth itself, they exert some toxic action on the bacterial cells and a part of the cells of the inoculum are killed (Experiment 5).[2]

## SUMMARY.

The growth of many pathogenic organisms in plain meat infusion broth is possible only when a large inoculum is used.

This requirement is much less strict when the broth cultures are

[2] It is realized that the oxidation-reduction potential of the broth is only one of many factors to be considered in the problem of initiation of growth. In the course of this work, several batches of plain broth prepared according to the standard method, have been found to be very poor for the growth of Pneumococcus and their value could not be enhanced by any of the treatments enumerated above.

It has been observed that samples of broth, several months old, become completely inadequate for the growth of Pneumococcus after they have been boiled, but that their value can be restored by addition of cysteine. This effect of boiling on very old broth is not as yet understood.

Recent experiments have shown that cystine could be replaced by equivalent concentrations of thioglycollic acid.

incubated (*a*) under anaerobic conditions, (*b*) in fresh media very recently boiled or autoclaved, (*c*) in fresh media reduced by means of hydrogen, or to which small amounts of cysteine or of blood have been added.

It is suggested that these findings can be accounted for by assuming that the bacterial species under consideration can multiply only in media the oxidation potential of which is below a critical value.

The favorable growth conditions obtained by the procedures enumerated above may be attributed to the establishment of a proper reduction potential in the medium; the same result is obtained by using a large inoculum, owing to the reducing properties of bacterial cells.

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# THE RELATION OF THE BACTERIOSTATIC ACTION OF CERTAIN DYES TO OXIDATION-REDUCTION PROCESSES.

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In a preceding paper (1) it has been suggested that the growth of certain facultative anaerobes is inhibited by the existence in the medium of substances with a high oxidation potential. Accurate studies on the critical potentials for different species of bacteria will require the use of media "poised" at definite values. Even then, the results will be significant only if it is demonstrated that there exists a condition of equilibrium between the different parts of the system under consideration.

Unfortunately, we have at our disposal only very few oxidation-reduction systems, the characteristics of which are known. Among these may be mentioned the color indicators of Clark. It seems that these indicators together with a few other systems, such as hemoglobin-methemoglobin, fumaric-succinic acid, hermidin, echinochrome and some bacterial pigments, might be used to "poise" the media at their own characteristic potentials. However, the use of this technique is complicated by the toxicity of some of the dyes for certain bacterial species. It appeared possible that, in some cases, this toxicity might be partly accounted for by the high oxidation potentials of the oxidized dyes. Were this hypothesis justified, it might give us a method of determining the critical potential of the bacterial species studied, by determining which of the dyes are toxic in the oxidized form, and not toxic in the reduced form.

The bactericidal and bacteriostatic actions of certain dyes on different groups of microorganisms has been known for a long time, but the mechanism of this action is still obscure; it is likely that the mechanism varies with each type of dyes and each group of organisms. An

extensive review of the subject has been recently presented by Sartorius (2), but without any special discussion of the fundamental principles involved.

Among the few facts which have been established in this respect may be mentioned the more or less complete parallelism between the bacteriostatic of triphenylmethanes and the Gram reaction (3). On the other hand, azine and azonium dyes, such as phenosafranine and Janus green are known to precipitate proteolytic enzymes (4) and to penetrate the living cell (5). A striking example of the differential toxicity of a dye for two closely related bacterial species has been described by Sherman and Albus (6). They showed that, while the growth of hemolytic streptococci of human and bovine origin is inhibited by the presence in the medium of even low concentrations of methylene blue, the cheese strains thrive in presence of much higher concentrations of the same dye. Later, Avery (7) found that the sample of methylene blue used contained Zn as impurity, and that Zn salts alone, in corresponding concentrations, would also inhibit the human and bovine strains. However, purified methylene blue retained the same property. Some time later, Brown (8) reported "that streptococci which were inhibited by methylene blue in the presence of oxygen grew well in media containing methylene blue which was reduced."

Burnet (9) studied the growth of certain organisms on acid fuchsin agar plates and found that the growth of "isolated" cells of *Staphylococcus aureus*, diphtheroid bacillus and Friedländer's bacillus was inhibited on such media. (Isolated cells were obtained by spreading a dilute suspension of the culture over the plate.) However, growth developed in the vicinity of colonies of *Staphylococcus aureus* obtained by local heavy inoculation. Burnet concluded from his experiments "that the fuchsin interferes directly with the production or utilization of peroxide by the organism. The presence of products of growth whose primary function is to destroy peroxide, protects isolated organisms against otherwise inhibiting concentrations of the dye. . . . . Fuchsin must act by rendering the organism sensitive to traces of peroxide produced in its own metabolism."

Although not dealing directly with the toxicity of dyes for bacterial cultures, the following three studies may be mentioned here.

W. M. Clark and associates (10) have indicated that the halogens lose the greater part of their toxicity when added under conditions which prevent the attainment of oxidation potentials positive to the indophenols. Voegtlin and collaborators (11), working with normal and cancer tissues, found that the "toxic action of dyes depends to some extent on their oxidizing power for reduced glutathione, and also on other features of their molecular structure" and demonstrated by toxicity tests "the existence of a biological antagonism between reduced glutathione and methylene blue." In the course of their studies on the intracellular oxidation-reduction potentials of *Amœba dubia*, Cohen, Chambers and

Reznikoff (12) observed that "the reductants of toxic oxidants were usually non-toxic" and that "the diazines were decidedly toxic."

The ultimate object of the following experiments was to find out whether indicators of oxidation-reduction can be used to determine the critical oxidation potential of the medium above which the growth of Pneumococcus and Streptococcus cannot take place. The problem which has been most directly considered here is an analysis of the mechanism by which such indicators exert a bacteriostatic action on these species.

TABLE I.

*List of Dyes* Used.*

| Name of oxidant | $E_0'$(pH = 7.4) (in volts) | rH |
|---|---|---|
| 2-Chloroindophenol (o-chlorophenol indophenol) | +0.233 | 21.8 |
| Indophenol (phenol indophenol) | +0.228 | 21.6 |
| 2-Methyl indophenol (o-cresol indophenol) | +0.195 | 20.5 |
| 1-Naphthol-2-sulfonate indophenol | +0.123 | 18.1 |
| Methylene blue | +0.011 | 14.4 |
| Janus green (green → pink) (approx.) | −0.02 | 13 |
| $K_4$ indigo tetrasulfonate | −0.046 | 12.5 |
| $K_3$ indigo trisulfonate | −0.081 | 11.3 |
| $K_2$ indigo disulfonate | −0.125 | 9.9 |
| K indigo monosulfonate | −0.182 | 7.5 |
| Janus green (pink ⇄ colorless) (approx.) | −0.26 | 5 |
| Neutral red (approx.) | −0.31 | 3.7 |
| Phenosafranine (approx.) | −0.525 | −3.5 |
| Litmus | ? | ? |
| Malachite green | ? | ? |

* The indophenols, the methylene blue, and 3 of the indigoes were obtained from La Motte Chemical Company. Indigo trisulfonate was obtained through the courtesy of Dr. B. Cohen of the Hygiene Laboratory, Washington, D. C. The other indicators were dyes used in the laboratory for staining and cytological work.

## EXPERIMENTAL.

### *Methods.*

12 hour plain broth cultures of the following organisms were used in the test

*Pneumococcus:* smooth virulent: Type I (1/200/4)
Type II (D/39/43)
Type III (A/66/69)

Rough avirulent derived from Type I (1/193/R)
Type II (D/39/R)
Type III (M/3/R)

*Streptococcus hæmolyticus:* human strains: L, S/43, S/23/Glossy, S/23/Matt
bovine strains: C/64, C/67
cheese strains: P, M

Since, in all cases, the results have been the same for all the strains of the same type of organism, only the results dealing with representatives of each type will be given.

The experiments were carried out in meat infusion broth, prepared 1 to 2 weeks before use.

The dyes used may be found in Table I.

The behavior of these dyes in plain broth has been studied in a preceding paper. It may be well to review briefly some of these results.

All the dyes studied function as reversible systems of oxidation-reduction, with the exception of the green → pink reduction of Janus green which is irreversible. The rH of all but two of them are known. When added to plain broth in concentrations not exceeding the reduction capacity of the medium, all indicators positive to indigo disulfonate, *i.e.* with an rH higher than 10, are reduced, if the system is kept under vaseline seal. The time of reduction for equimolecular concentrations of the dyes increases progressively as the rH becomes more negative. Although no electrometric measurements are available for malachite green and litmus, the fact that they were not reduced by plain broth suggests that their rH is smaller than that of indigo disulfonate.

Many of the indicators, when added to broth, are rapidly decomposed under aerobic conditions, but remain stable under vaseline seal; the indophenols are especially unstable (particularly methyl indophenol, and phenol indophenol).

The dye solutions were autoclaved, except the solutions of indophenols which were prepared (with aseptic technique) and proved to be sterile.

### *Aerobic Growth of Pneumococcus and Hemolytic Streptococcus in the Presence of Oxidized Dyes.*

*Experiment 1.*—To a series of test-tubes each containing 5 cc. of broth, sufficient amounts of the different dyes were added to give final concentrations of 0.001 M, 0.003 M, 0.0001 M. The tubes were inoculated with 0.1 to 0.01 cc. of

TABLE II.

*Growth of Pneumococcus and Hemolytic Streptococci in the Presence of rH Indicators.*

| Indicator | | Pneumococcus R strain derived from Type II (D/39/R) | | *Streptococcus hæmolyticus.* Human strain. L | | *Streptococcus hæmolyticus.* Cheese strain P | |
|---|---|---|---|---|---|---|---|
| | | Inoculum cc. | | Inoculum cc. | | Inoculum cc. | |
| | | $10^{-1}$ | $10^{-2}$ | $10^{-1}$ | $10^{-2}$ | $10^{-1}$ | $10^{-2}$ |
| | M | | | | | | |
| 2-Chloroindophenol | 0.001 | − | − | − | − | +* | +† |
| | .0003 | − | − | − | − | + | + |
| | .0001 | + | − | − | − | + | + |
| Phenol indophenol | .001 | − | − | − | − | +† | +† |
| | .0003 | − | − | − | − | + | + |
| | .0001 | + | − | − | − | + | + |
| 2-Methyl indophenol | .001 | Erratic results due to breaking down of the dye in the broth | | | | + | + |
| | .0003 | | | | | + | + |
| | .0001 | | | | | + | + |
| 2-Sulfonate-1-naphthol indophenol | .001 | − | − | − | − | + | + |
| | .0003 | − | − | + | − | + | + |
| | .0001 | + | − | + | + | + | + |
| Methylene blue | .001 | − | − | − | − | + | + |
| | .0003 | − | − | − | − | + | + |
| | .0001 | + | − | − | − | + | + |
| Janus green | .001 | − | − | − | − | + | + |
| | .0003 | − | − | − | − | − | − |
| | .0001 | − | − | − | − | − | − |
| Indigo tetrasulfonate | .001 | + | + | + | + | + | + |
| | .0003 | + | + | + | + | + | + |
| | .0001 | + | + | + | + | + | + |
| Indigo trisulfonate | .001 | + | + | + | + | + | + |
| | .0003 | + | + | + | + | + | + |
| | .0001 | + | + | + | + | + | + |

In this table + indicates that growth developed in 24 hours; − indicates no growth.

* The dyes are arranged in the order of the electromotive series (see Table I).

† The growth of the cheese strains of *Streptococcus hæmolyticus* was delayed in the presence of 2-chloroindophenol and phenol indophenol but finally developed in 36 hours.

TABLE II—*Concluded.*

| | | Pneumococcus R strain derived from Type II (D/39/R) | | *Streptococcus hæmolyticus.* Human strain L | | *Streptococcus hæmolyticus.* Cheese strain P | |
|---|---|---|---|---|---|---|---|
| | | Inoculum cc. | | Inoculum cc. | | Inoculum cc. | |
| | | $10^{-1}$ | $10^{-2}$ | $10^{-1}$ | $10^{-2}$ | $10^{-1}$ | $10^{-2}$ |
| | M | | | | | | |
| Indigo disulfonate | .001 | + | + | + | + | + | + |
| | .0003 | + | + | + | + | + | + |
| | .0001 | + | + | + | + | + | + |
| Indigo monosulfonate | .001 | + | + | + | + | + | + |
| | .0003 | + | + | + | + | + | + |
| | .0001 | + | + | + | + | + | + |
| Neutral red | .001 | − | − | − | − | − | − |
| | .0003 | − | − | − | − | + | − |
| | .0001 | − | − | − | − | + | + |
| Phenosafranine | .001 | − | − | − | − | − | − |
| | .0003 | − | − | − | − | + | + |
| | .0001 | − | − | − | − | + | + |
| Malachite green | .001 | + | + | + | + | + | + |
| | .003 | + | + | + | + | + | + |
| | .0001 | + | + | + | + | + | + |
| Litmus | ? | + | + | + | + | + | + |

TABLE III.

*Time Required for the Growth of Cheese Strain (P) of Hemolytic Streptococcus to Develop in the Presence of Indophenols.*

| Dye | Concentration | Inoculum cc. | | | |
|---|---|---|---|---|---|
| | | $10^{-3}$ | $10^{-4}$ | $10^{-5}$ | $10^{-6}$ |
| | M | *hrs.* | *hrs.* | *hrs.* | *hrs.* |
| 2-Chloroindophenol | 0.001 | 28 | 48 | 48 | 48 |
| Phenol indophenol | 0.001 | 28 | 28 | 28 | 28 |
| 1-Naphthol-2-sulfonate indophenol | 0.001 | 12 | 18 | 20 | 20 |

the various cultures and growth was recorded after 36 hours incubation at 37°C. Typical protocols are given in Table II.

The results were definite except in the case of the cheese strain of *Streptococcus hæmolyticus*, the growth of which was retarded in the presence of some of the indophenols but finally developed. The question was investigated further by using smaller inocula of this strain (Table III).

Judging from the color of the solution, a good deal of the 2-chloroindophenol was decomposed in 48 hours, so that it may be concluded that the growth of cheese strains of *Streptococcus hæmolyticus* is at least partially inhibited by 2-chloroindophenol, and also perhaps by phenol indophenol.

Summarizing the results of Tables II and III, it appears that the growth of Pneumococcus and of human strains of *Streptococcus hæmolyticus* is inhibited by all the dyes positive to indigo tetrasulfonate (*i.e.* with an rH higher than 12.5), and by neutral red and phenosafranine. The cheese strains of *Streptococcus hæmolyticus* are inhibited only and more or less completely by the most positive indophenols and by Janus green, neutral red and phenosafranine.

*Toxic Action of Some of the Oxidized Dyes on Cultures of Streptococcus hæmolyticus.*

It is probable that the effect of the addition of dyes to broth is manifold; it is known for instance, that bacteriostatic and bactericidal action of dyes do not always run parallel (3). Before attempting to find whether the bacteriostatic action of the oxidized forms of rH indicators is due in many cases to a "poising" of the medium at a high oxidation potential, it is necessary to eliminate from our tests those indicators which can be shown to exhibit a primary toxicity, not related to oxidation-reduction phenomena. While the object of Experiment 1 was to establish which of the dyes would prevent cell multiplication, Experiment 2 served to establish whether any of these dyes would be toxic for the cells. This was tested by adding dyes to a fully developed culture and studying their effect.

*Experiment 2.*—12 hour broth cultures of *Streptococcus hæmolyticus* were transferred to test-tubes in amounts of 2 cc. per tube; sufficient amounts of the

dyes were then added to give a final concentration of 0.005 M. After exposure of the cultures to the dyes for different lengths of time, subcultures were made on blood agar plate to determine the viability. The test was not performed with Pneumococcus because the cells of this organism autolyze too rapidly.

The results of Experiment 2 are given in Table IV.

These results indicate that, under the conditions of the experiment,

TABLE IV.

*Toxic Action of rH Indicators* on Streptococcus hæmolyticus.*

| Dyes in 0.005 M concentration | *Streptococcus hæmolyticus* | | | | | | |
|---|---|---|---|---|---|---|---|
| | Human strain | | | | | Cheese strain | |
| | Time of exposure (hrs.) | | | | | Time of exposure (hrs.) | |
| | 2 | 4 | 7 | 10 | 28 | 10 | 28 |
| 2-Chloroindophenol | + | + | + | + | + | + | + |
| Phenol indophenol | + | + | + | + | + | + | + |
| 1-Naphthol-2-sulfonate indophenol | + | + | + | + | + | + | + |
| Methylene blue | + | + | + | + | + | + | + |
| Janus green | − | − | − | − | − | ± | − |
| Indigo tetrasulfonate | + | + | + | + | + | + | + |
| Indigo trisulfonate | + | + | + | + | + | + | + |
| Indigo disulfonate | + | + | + | + | + | + | + |
| Indigo monosulfonate | + | + | + | + | + | + | + |
| Neutral red | + | ± | − | − | − | + | − |
| Phenosafranine | + | + | + | + | + | + | + |
| Litmus | + | + | + | + | + | + | + |
| Malachite green | + | + | + | + | + | + | + |

* The dyes are arranged in the order of the electromotive series (see Table I).

− indicates no growth on blood agar, ± indicates poor growth on blood agar, + indicates good growth on blood agar.

Janus green, neutral red and phenosafranine exerted a definite toxic action on the cells of *Streptococcus hæmolyticus*. On the contrary, the cells remained alive much longer in the indophenols, methylene blue, the indigoes, malachite green and litmus. Further work was therefore limited to this latter group of dyes.

*Growth of Pneumococcus and Hemolytic Streptococcus in the Presence of Reduced Indophenols.*

If the bacteriostatic action of the oxidized indophenols and methylene blue is due to the fact that they "poise" the medium at a high oxidation potential, the reduced forms of these dyes should be inactive.

*Experiment 3.*—The bacteriostatic action of the reduced dyes was tested in the apparatus represented in Fig. 1. This apparatus is a slight modification of the one described in a previous article (1) Fig. 2, the tube through which the hydrogen enters the system being shorter in order to avoid bubbling gas through the culture.

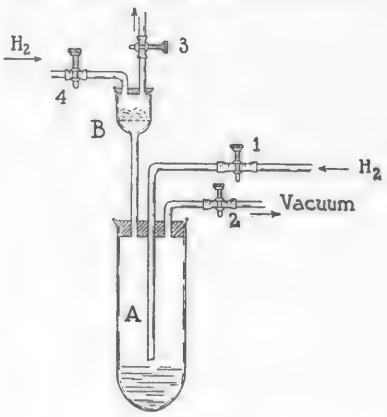


FIG. 1.

The experiment was carried out as follows:

10 cc. portions of recently boiled broth were transferred to each of 36 large tubes; the reduced dyes were added to 12 of them which were filled with the reduction apparatus (Fig. 1); 12 other tubes were treated with the oxidized dyes and the last 6 did not receive any treatment. The dyes (2-chloroindophenol and 1-naphthol-2-sulfonate indophenol) were used in concentrations of 0.0005 M. All the tubes had been inoculated before treatment. Growth was recorded after 24 hours incubation at 37°C. (Table V).

This experiment confirms the complete inhibition of growth of Pneumococcus and human strains of *Streptococcus hæmolyticus* by the oxidized indophenols and the partial inhibition of the cheese strains by the oxidized 2-chloroindophenol. On the contrary, all the cultures grew well in the presence of the reduced dyes.

*The Effect of the Addition of Oxidized Dyes to Plain Broth on the Growth of Pneumococcus and Streptococcus hæmolyticus Incubated under Anaerobic Conditions.*

It has been shown (13) that the indophenols and methylene blue are reduced by plain broth when the system is kept under vaseline seal. In consideration of the results of Experiments 3, it appears likely there-

fore that the bacteriostatic action of the dyes would be greatly decreased under vaseline seal.

It is also known that methylene blue is reduced in plain broth when the mixture is kept in anaerobic jars. As was to be expected, the indophenols are also reduced under such conditions.

*Experiment 4.*—The toxicity of methylene blue and indophenols was tested under vaseline seal and in anaerobic jars. In the course of the experiments, it was found preferable to use smaller concentrations of methylene blue than of indophenols for the two following reasons; (*a*) methylene blue is reduced only very slowly in high concentration, (*b*) methylene white being but little soluble precipitates out.

TABLE V.

*Influence of the Addition of Oxidized and Reduced Indophenols on the Growth of Pneumococcus and Hemolytic Streptococci.*

| Dyes in 0.0005 M concentration | Pneumococcus | | *Streptococcus hæmolyticus* | | | |
|---|---|---|---|---|---|---|
| | Inoculum cc. | | Human strains | | Cheese strains | |
| | | | Inoculum cc. | | Inoculum cc. | |
| | $10^{-1}$ | $10^{-2}$ | $10^{-1}$ | $10^{-2}$ | $10^{-1}$* | $10^{-2}$* |
| 2-Chloroindophenol, oxidized | − | − | − | − | + | − |
| 2-Chloroindophenol, reduced | + | + | + | + | + | + |
| 1-Naphthol-2-sulfonate indophenol, oxidized | − | − | − | − | + | − |
| 1-Naphthol-2-sulfonate indophenol, reduced | + | + | + | + | + | + |
| Controls | + | + | + | + | + | + |

* As already pointed out in Experiment 1, the bacteriostatic action of oxidized indophenols on cheese strains of *Streptococcus hæmolyticus* can be recognized only when very small inocula are used.

On the other hand, both the reduced and oxidized forms of the indophenols are very soluble and the oxidized forms are reduced rapidly even in concentrations as high as 0.0005 M; furthermore, these dyes decompose rapidly under aerobic conditions in the presence of broth, and it is necessary to use high concentrations to obtain sharp results.

In this experiment, the indophenols and indigoes were used in 0.0005 M concentrations and methylene blue in 0.00006 M concentrations.

The broths used were 6, 3 and 9 days old, respectively. The cultures were Pneumococcus (D/39/R), *Streptococcus hæmolyticus*, human strain L and cheese strain P. The size of inocula used and the results are given in Tables VI, VII and VIII. The tubes marked "vaseline" were sealed with a 2 cm. layer of vaseline; the anaerobic jars were incubated for 48 hours before being opened.

The results of Tables VI, VII and VIII constitute a new demonstration of the bacteriostatic action of the oxidized forms of methylene blue and the indophenols on Pneumococcus and human strains of

TABLE VI.

*Growth of Pneumococcus (D/39/R) in the Presence of Dyes, under Aerobic and Anaerobic Conditions.*

| Dye | Aerobic | | | | Anaerobic | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Vaseline seal | | | | | | Jar | | | | | |
| | Inoculum cc. | | | | Inoculum cc. | | | | | | Inoculum cc. | | | | | |
| | $10^{-1}$ | $10^{-2}$ | $10^{-3}$ | $10^{-4}$ | $10^{-1}$ | $10^{-2}$ | $10^{-3}$ | $10^{-4}$ | $10^{-5}$ | $10^{-6}$ | $10^{-1}$ | $10^{-2}$ | $10^{-3}$ | $10^{-4}$ | $10^{-5}$ | $10^{-6}$ |
| 2-Chloroindophenol 0.0005 M | − | − | − | − | + | + | + | + | − | − | + | + | + | − | − | − |
| 1-Naphthol-2-sulfonate indophenol 0.0005 M | − | − | − | − | + | + | + | − | − | − | + | + | − | − | − | − |
| Methylene blue 0.00006 M | − | − | − | − | + | + | − | − | − | − | + | + | + | + | − | − |
| Indigo tetrasulfonate 0.0005 M | + | + | − | − | + | + | + | + | + | − | + | + | + | − | − | − |
| Plain broth | + | + | − | − | + | + | + | + | − | − | + | + | + | − | − | − |

+ indicates that growth developed, − indicates no growth developed.

TABLE VII.

*Growth of Human Strains (L) of Streptococcus hæmolyticus in the Presence of Dyes, under Aerobic and Anaerobic Conditions.*

| Dye | Aerobic | | | | Anaerobic | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Vaseline seal | | | | | | Jar | | | | | |
| | Inoculum cc. | | | | Inoculum cc. | | | | | | Inoculum cc. | | | | | |
| | $10^{-1}$ | $10^{-2}$ | $10^{-3}$ | $10^{-4}$ | $10^{-1}$ | $10^{-2}$ | $10^{-3}$ | $10^{-4}$ | $10^{-5}$ | $10^{-6}$ | $10^{-1}$ | $10^{-2}$ | $10^{-3}$ | $10^{-4}$ | $10^{-5}$ | $10^{-6}$ |
| 2-Chloroindophenol 0.0005 M | − | − | − | − | + | + | + | − | − | − | + | + | + | − | − | − |
| 1-Naphthol-2-sulfonate indophenol 0.0005 M | − | − | − | − | + | + | − | − | − | − | + | − | − | − | − | − |
| Methylene blue 0.00006 M | − | − | − | − | + | + | + | − | − | − | + | + | + | − | − | − |
| Indigo tetrasulfonate 0.0005 M | + | + | + | − | + | + | + | + | + | + | + | + | + | + | − | − |
| Plain broth | + | + | + | − | + | + | + | + | + | − | + | + | + | + | − | − |

+ indicates that growth developed, − indicates no growth developed.

*Streptococcus hæmolyticus;* as expected, the cheese strain of *Streptococcus hæmolyticus* was inhibited only by high concentrations of 2-chloroindophenol and when the inoculum was small. Under anaerobic

conditions (vaseline seal or anaerobic jar), methylene blue and the indophenols lost a great part of their bacteriostatic action. In the tubes kept under vaseline seal, it was possible to observe that, in the

TABLE VIII.

*Growth of Cheese Strain (P) of Streptococcus hæmolyticus in the Presence of Dyes, under Aerobic and Anaerobic Conditions.*

| Dye | Aerobic | | | | | Anaerobic | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Vaseline seal | | | | | Jar | | | | |
| | Inoculum cc. | | | | | Inoculum cc. | | | | | Inoculum cc. | | | | |
| | $10^{-3}$ | $10^{-4}$ | $10^{-5}$ | $10^{-6}$ | $10^{-7}$ | $10^{-3}$ | $10^{-4}$ | $10^{-5}$ | $10^{-6}$ | $10^{-7}$ | $10^{-3}$ | $10^{-4}$ | $10^{-5}$ | $10^{-6}$ | $10^{-7}$ |
| 2-Chloroindophenol 0.001 M | − | − | − | − | − | + | + | + | + | + | + | + | + | + | − |
| 1-Naphthol-2-sulfonate indophenol 0.001 M | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Plain broth | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |

+ indicates that growth developed, − indicates no growth developed.

TABLE IX.

*Influence of 0.00002 M Methylene Blue on the Growth of Type II Pneumococcus (D/39/3) in "Boiled" and "Unboiled" Broth under Aerobic and Anaerobic Conditions.*

| Inoculum | Aerobic | | | | Anaerobic Jar | | | |
|---|---|---|---|---|---|---|---|---|
| | "Unboiled" broth | | "Boiled" broth | | "Unboiled" broth | | "Boiled" broth | |
| | No methylene blue | Methylene blue | No methylene blue | Methylene blue | No methylene blue | Methylene blue | No methylene blue | Methylene blue |
| *cc.* | | | | | | | | |
| $10^{-1}$ | + | + | + | + | + | + | + | + |
| $10^{-2}$ | + | − | + | + | + | + | + | + |
| $10^{-3}$ | − | − | + | − | − | + | + | + |
| $10^{-4}$ | − | − | + | − | − | + | + | + |
| $10^{-5}$ | − | − | + | − | − | + | + | + |
| $10^{-6}$ | − | − | + | − | − | − | + | + |

+ indicates that growth developed, − indicates no growth developed.

presence of these dyes, growth did not develop as long as the dye was not reduced. On the contrary, growth developed in the presence of the oxidized form of indigo tetrasulfonate.

The unexpected observation has been made that the initiation of growth of Pneumococcus is facilitated in the anaerobic jar when traces of methylene blue are added to the broth. The most characteristic example of such an effect is given in Table IX.

*Experiment 5.*—One half of the broth used in this experiment had been boiled immediately before inoculation. The "boiled" and "unboiled" samples were divided again into two series; one of them was inoculated as such, the other one only after addition of 0.00002 M methylene blue. The inoculated tubes were incubated either under aerobic conditions, or in an anaerobic jar. The observations were made after 36 hours incubation (Table IX).

Similar results have been obtained with 6 different strains of Pneumococcus. They confirm that methylene blue is not toxic, when reduced in the broth. In the anaerobic jar, growth developed only with $10^{-1}$ and $10^{-2}$ cc. inoculum in the plain "unboiled" broth, while it took place with as little as $10^{-5}$ cc. inoculum in the presence of 0.00002 M methylene blue. However, even such a minute concentration of the dye is bactericidal in the oxidized form, as appears from the results obtained under aerobic conditions. (This is especially striking in the case of "boiled" broth.) The growth of very small inocula in the presence of traces of methylene blue in the anaerobic jar must be due therefore to an indirect effect. It can be explained by assuming that methylene blue, being such an actively reversible system, does accelerate the reduction of the broth itself.

*Comparative Growth of Human and Cheese Strains of Streptococcus hæmolyticus in Plain Broth.*

It has been established that the growth of human strains of *Streptococcus hæmolyticus* is completely checked by the presence in the medium of the oxidized forms of methylene blue and the indophenols, while the growth of the cheese strains of *Streptococcus hæmolyticus* is inhibited only by the 2-chloroindophenol, the most positive of the indicators tested. On the other hand, it has been suggested elsewhere (1) that the growth of small inocula of human strains of *Streptococcus hæmolyticus* may be inhibited by the presence of oxidized substances in plain broth. It was interesting to compare the growth of small inocula of human and cheese strains of *Streptococcus hæmolyticus* in this medium.

*Experiment 6.*—Varying amounts of 12 hour cultures of human (L) and cheese (P) strains of *Streptococcus hæmolyticus* were seeded into test-tubes containing 5 cc. of plain broth (1 week old). Growth was recorded after 24 hours (Table X).

The results of Table X indicate that, while a fairly large inoculum is necessary to insure the growth of human strains of *Streptococcus hæmolyticus* in plain broth, the cheese strains develop in the same medium following inoculation of a very few cells only.

## DISCUSSION.

Janus green, neutral red and phenosafranine form a special class among the indicators of oxidation-reduction potential used in this work.

TABLE X.

*Growth of Human (L) and Cheese (P) Strains of Streptococcus hæmolyticus in Plain Broth.*

| *Streptococcus hæmolyticus* | Inoculum cc. | | | | | | |
|---|---|---|---|---|---|---|---|
| | $10^{-1}$ | $10^{-2}$ | $10^{-3}$ | $10^{-4}$ | $10^{-5}$ | $10^{-6}$ | $10^{-7}$ |
| Cheese strain P..... | + | + | + | + | + | + | + |
| Human strain L..... | + | + | − | − | − | − | − |

+ indicates that growth developed, − indicates no growth developed.

They rapidly kill cultures of hemolytic streptococci to which they are added in sufficiently high concentrations. These cultures remain alive for a longer time when put in contact with the same concentrations of the other indicators. This toxic action of Janus green, neutral red and phenosafranine is not related to phenomena of oxidation-reduction. The discussion will therefore be limited to the other indicators.

The results concerning the bacteriostatic action of the indophenols, methylene blue, the indigoes, malachite green and litmus on Pneumococcus and *Streptococcus hæmolyticus* are summarized in Table XI.

In Table XI, the dyes are arranged in the order of their oxidation-reduction potentials (electromotive series). It is apparent that Pneumococcus and human strains of *Streptococcus hæmolyticus* are inhibited only by the indophenols and methylene blue; *i.e.* by the dyes having an rH higher than 12.5. The cheese strains of *Streptococcus*

*hæmolyticus* are inhibited by 2-chloroindophenol and perhaps phenol indophenol, but not by the others, indicating that their "bacteriostatic potential" is above rH 21.

The bacteriostatic action of the indophenols and methylene blue is completely or partly corrected when (*a*) they are added to the medium in the reduced form, (*b*) the mixture broth-dye is incubated under anaerobic conditions (the concentration of dyes being low enough to allow their reduction in a reasonable time).

TABLE XI.

*Growth of Pneumococcus and Hemolytic Streptococci in the Presence of Different rH Indicators Used in the Oxidized Form under Aerobic Conditions.*

| Dye* | Pneumococcus | *Streptococcus hæmolyticus* | |
|---|---|---|---|
| | | Human strain | Cheese strain |
| 2-Chloroindophenol | − | − | +− |
| Phenolindophenol | − | − | +− (?) |
| 1-Naphthol-2-sulfonate indophenol | − | − | + |
| Methylene blue | − | − | + |
| Indigo tetrasulfonate | + | + | + |
| Indigo trisulfonate | + | + | + |
| Indigo disulfonate | + | + | + |
| Indigo monosulfonate | + | + | + |
| Malachite green | + | + | + |
| Litmus | + | + | + |

* The dyes are arranged in the order of the electromotive series (see Table I).
− indicates complete inhibition of growth, +− indicates partial inhibition of growth, + indicates normal growth.

It has already been suggested that, before an organism can multiply, the medium in which it is present must reach a critical reduction potential. It is attractive to suppose that, for Pneumococcus and human strains of hemolytic streptococcus, the critical potential is between indigo tetrasulfonate (rH = 12.5) and methylene blue (rH = 14.4) while it is somewhere below the chloroindophenol for the cheese strains of *Streptococcus hæmoyticus*. The corollary of such a view is that all the dyes or other substances with an oxidation potential higher than that of indigo tetrasulfonate would be bacteriostatic for Pneumococcus and human strains of hemolytic streptococcus, while

only the ones positive to phenol indophenol would have such as action on cheese strains of hemolytic streptococcus (provided that the dye is not primarily toxic and that conditions of equilibrium exist between the dye and the culture).

In terms of this hypothesis, the large inocula required for obtaining a growth of Pneumococcus and of human strains of *Streptococcus hæmolyticus* in plain broth, serve the purpose of reducing the broth to a potential (rH = 13) corresponding to reduced methylene blue. The cheese strains of *Streptococcus hæmolyticus* on the contrary grow with such a small inoculum because the potential of aerated broth is within the range in which these organisms can multiply. In fact, the oxidation potential of the broth is certainly not much higher than that of oxidized 2-chloroindophenol, since the dye remains reduced for a long time in broth that has been recently boiled, and aerated afterwards. In fact, 2-chloroindophenol is the only dye tested which exerts a definite bacteriostatic action, and even then, this action is only partial, indicating that we are just on the border of the critical potential.

The fact that the dyes are not toxic in the reduced form is of course in agreement with the hypothesis.

Let us now consider a few of the objections that may be made to this point of view. When the dyes are added in the reduced form, they remain reduced in the medium only if the broth is at an oxidation potential below, or equal to, that of the reduced dye. On the other hand, if oxidized indophenols or methylene blue are added to reduced broth, and the system kept under aerobic conditions to prevent reduction of the dye, it is likely that the broth will oxidize rapidly. Consequently, when we are dealing with oxidized or reduced dye, we are dealing at the same time with oxidized or reduced broth. As we know that the condition of the broth is of the greatest importance for initiation of growth (1), it is hard to know whether the dye is no longer toxic because present in the reduced form or only because the reduced broth is a more favorable medium. To this objection one can answer by pointing out that according to the hypothesis, both oxidized broth and the oxidized indicators under consideration are bacteriostatic, *not as broth or dye*, but by virtue of their *oxidizing intensity*. In both cases, fundamentally, the mechanism of action is the same, and a solution of the problem will be possible only when we have at our disposal media which do not function themselves as active systems of oxidation reduction.

Another weak point is that toxicity stops exactly with the indigoes. Is it not possible that toxicity is related, not to the oxidation-reduction characteristics of the dyes, but to their structure? A convincing proof would be to find an organism inhibited only by some of the indigoes, and not by the ones with a more negative rH. An interesting evidence of this sort is supplied by the fact that cheese strains of *Streptococcus hæmolyticus* are inhibited only by the most positive of the indophenols tested. In that respect, it would be important to test the action of other indophenols.

As they stand, our experiments do not supply any fact to contradict the hypothesis that methylene blue and oxidized indophenols are bacteriostatic because they "poise" the medium at an oxidation potential outside the range in which Pneumococcus and human and bovine strains of hemolytic streptococcus can grow. The problem of the mechanism whereby such a medium, poised at a high oxidation potential, becomes bacteriostatic for certain species, can be considered from at least two points of view. On the one hand, we may suppose that the presence of oxidized dyes in the medium results in such a change in the condition of some of the constitutents of the broth, that they are no longer available for growth. On the other hand, the action of the dye may be not on the broth, but on the bacterial cells themselves. We know for instance, that the pneumococcus cell contains a dual system of oxidation-reduction consisting of a thermolabile cellular constituent, and thermostable autoxidizable substances which can be removed by washing (14). By means of this system, the Pneumococcus cell is able to carry on a series of oxidations and reductions, the thermostable components becoming reversibly reduced or oxidized in the process, while, under aerobic conditions, the cellular component is inactivated by oxidation. It may be considered that the oxidized dyes bring the autoxidizable substances, or the thermolabile cellular constituent, to an oxidation potential where they are no longer adjusted to the metabolic activities of the cell.

Whatever its explanation may be, the fact remains that the indicators studied are much less toxic under anaerobic than under aerobic conditions. The significance of this observation for the use of dyes in therapeutics is evident since their action "*in vitro*," under aerobic conditions, is little indication of what it will be in the presence of living tissues which are known to be active reducing agents. It is to be

expected that some dyes will be very effective when used on superficial wounds, but not when injected.

Undoubtedly, the mode of action of dyes is manifold, but the observations presented above may help in the analysis of this action, by pointing to one of the factors to be controlled.

## SUMMARY.

Oxidized indophenols and methylene blue are bacteriostatic for Pneumococcus and hemolytic streptococci of human and bovine origin, while the indigoes, malachite green and litmus are not toxic.

2-Chloroindophenol, the most positive of the indicators of oxidation-reduction potentials used, is also the only one to have a bacteriostatic action on cheese strains of *Streptococcus hæmolyticus*.

Methylene blue and the indophenols are no longer bacteriostatic when present in a reduced form in a medium capable of maintaining them in such a condition.

A comparison of these results with the growth in plain broth of the organisms studied suggests that the "inhibiting" dyes "poise" the medium at an oxidation potential outside the range in which the inhibited organisms can grow.

The validity of this hypothesis is discussed.

The significance of these observations for the use of dyes in therapeutics is considered.

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# STUDIES ON BACILLUS TYPHOSUS TOXIC SUBSTANCES.

## II. The Effect of Sera upon the Factors Determining Local Skin Reactivity to Filtrates of Bacillus Typhosus Cultures.*

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In the previous communication (1) a local skin reactivity to *B. typhosus* culture filtrates was described. The reactivity was induced by injection of the filtrate into the skin. If 24 hours later an intravenous injection of the same filtrate was given to the rabbit, there appeared an extremely severe hemorrhagic necrosis at the site of the previous injection. The mechanism is not fully understood as yet since no complete experimental comparison has been made between this phenomenon and the manifestations of bacterial allergy. There were found, however, certain features which, *considered together*, distinguish this phenomenon from the known phenomena of bacterial hypersusceptibility and from the Arthus phenomenon. These features are: the local reactivity; the short incubation period necessary for local preparation of the skin; the short duration of the state of reactivity; the ability to induce the reactivity by a single injection; and the necessity to make the second injection of the toxic agent by the intravenous route.

The present paper deals with the relation of the specific antisera to the skin-preparatory factors of the phenomenon above described. There was a double purpose in these studies, first, to elucidate the mechanism of the phenomenon, and secondly, to determine whether specific sera neutralized the preparatory factors, and if so, whether this could be advantageously applied to titration of the neutralizing

* A preliminary report of this work was presented before the Society of American Bacteriologists, Richmond, Virginia, December, 1928.

properties of the sera. In pursuance of this plan the effect of homologous, normal and heterologous sera was studied.

### *Methods.*

*Preparation of Immune Antisera.*—Most sera were prepared by the injection of toxic culture filtrates. Since a considerable number of the rabbits died from intensive immunization with these untreated filtrates, some animals were injected at the beginning of the immunization with autoclaved filtrates and later with filtrates heated in the Arnold steam sterilizer as also with filtrates heated at 60° for 1–2 hours, and finally with potent toxic filtrates. Some animals received injections of whole bacteria, first heated to 60° for 2 hours, then live bacteria.

Most of the injections were made intravenously, but in some instances the subcutaneous route was employed. They were usually made every week for 3 or 4 successive days for a period of 6–7 weeks. The final bleedings were made about 10 days after the last injection or earlier if the animals appeared sick.

Heterologous sera were similarly prepared. *B. typhosus*, Para A and B, and Shiga bacillus sera employed in some experiments, were prepared in the New York Board of Health Laboratories by immunization of horses. Other animals employed in this work for immunization purposes were goats and rabbits.

*Technique of the Neutralization Experiments.*—The toxic filtrates were mixed with sera in the desired proportions and the mixtures were incubated in the water bath at 37° for 1 hour. Precipitations were always recorded. Immediately before injection the tubes were shaken and the fluid together with the precipitate was used for injections. The skin of the abdomen of rabbits was prepared, as described in the first paper of this series (1), and the mixture injected into various areas, usually the lower right and upper and lower left areas of the shaven skin of the abdomen. The upper right area of the skin of the abdomen of each rabbit was injected with the toxic filtrate alone, previously diluted with 0.9 per cent sodium chloride solution and kept in the water bath at 37° for 1 hour. Sometimes the order of the injections was changed. 24 hours afterwards, the *B. typhosus* culture filtrate was injected intravenously and readings of the reactions were made 4–5 hours after the intravenous injections. It may be stated here that the specific sera had no obvious neutralizing effect upon the erythema frequently produced by the skin injections alone. Data on this point are, therefore, omitted from the experimental part of this paper.

## EXPERIMENTAL.

### *A. Effect of Immune Sera upon the Factors Determining Local Skin Reactivity.*

About 16 per cent of rabbits in this series gave no reaction and are accordingly omitted from Table I which represents a summary of the

neutralization experiments performed according to the technique described above. Reports on the rabbits that died earlier than 3 hours after the intravenous injection are also omitted for purposes of clarity.

The doses of *B. typhosus* culture filtrates used for the skin and intravenous injections were kept approximately constant.[1] Since some of the rabbits showed from time to time positive reactions elicited by neutralized mixtures, it was necessary to employ several rabbits for each serum tested. Moreover, since high concentrations of serum

TABLE I.

*Neutralization of B. typhosus Skin-Preparatory Factors by Immune Sera.*

| Sera titrated | Total number of rabbits | Number showing neutralization with the sera; and dilutions of the latter | | | | | Number showing no neutralization |
|---|---|---|---|---|---|---|---|
| | | 1:2000 | 1:200 | 1:100 | 1:20 | 1:2 | |
| No. 446 | 13 | 6 | 4 | — | 1 | — | 2 |
| No. 90 | 10 | 3 | 3 | — | — | — | 4 |
| No. 59 | 10 | 2 | 4 | — | 1 | 1 | 2 |
| Typhoid horse serum | 9 | — | 6 | — | 1 | — | 2 |
| No. 78 | 10 | — | 4 | 2 | 1 | — | 3 |
| No. 165. Goat typhoid serum | 10 | — | — | — | 3 | 3 | 4 |

Sera 446, 90, 59, 78 and Goat 165 were prepared by immunization with *B. typhosus* culture filtrates. Typhoid horse serum obtained from the New York Board of Health was prepared by immunization with dead and live *B. typhosus*. In all of these rabbits there was a positive reaction in the control areas.

occasionally failed to neutralize (prozone?), whereas higher dilutions of the same serum and in the same rabbit gave complete neutralization the use of several dilutions of serum was necessitated.

The results can be summarized as follows:

There are two types of response to the injection of mixtures of specific sera with the skin-preparatory factors, as demonstrated by

[1] The *B. typhosus* culture filtrate (see page 600 for preparation) was diluted 1:2 for skin injection. 0.25 cc. of filtrate in this dilution represents two minimal skin-preparatory doses, each of sufficient strength to prepare for a severe hemorrhagic necrosis. The intravenous dose was 2 cc. per kilo of body weight in most instances.

intravenous injections 24 hours later, namely, neutralization of the reaction and non-neutralization. Out of a total of 316 tests, there were 135 neutralizations by sera in various dilutions and 181 failures to neutralize. This brings out the fact that neutralization of *B. typhosus* skin-preparatory factors by specific sera can be induced at will in a large proportion of experimental rabbits (about 42.5 per cent).

Varying the site of the injection was without influence on the result.

After establishing definitely the fact that neutralization of skin-preparatory factors by specific sera is possible it appeared desirable to analyze the results further in order to determine whether the phenomenon could be advantageously used for titrating the neutralizing power of specific sera. The data obtained with a given immune serum in a large number of rabbits were examined more closely. As is seen from Table I, in six rabbits receiving *B. typhosus* horse serum complete neutralization was obtained in dilution of 1:200, in one rabbit in dilution 1:20, while in two rabbits there was no neutralization. When analysis of the sort is practiced in connection with the other sera it becomes evident that there is a definite variation in the response of different rabbits to the skin-preparatory effect of serum-filtrate mixtures and that the rabbits showing these varying responses can be classified under three groups, namely, those which yield completely neutralized reactions with the highest dilutions (HN) of serum,[2] those which give rise to completely neutralized reactions only with sera in lower dilutions (LN) and those which show non-neutralized reactions (NN).

Possibly some non-neutralizable fraction of the culture filtrate to which certain rabbits are particularly susceptible may account for the occurrence of non-neutralization. Furthermore, it appears from Table I that the potency of a given serum bears a mathematical ratio to the reactions obtained in the three groups of rabbits. Thus if complete neutralization is obtained, for example, in dilution 1:2000 in HN rabbits, namely, in those showing the highest titer of neutralization, the titer in LN rabbits is likely to be 1:200. In the cases in

[2] In a preliminary report (3) recently published, HN, LN and NN were called CN, PN and NN respectively. It is now felt that the nomenclature here suggested is more satisfactory.

which the HN is 1:200 the LN titer is only likely to be 1:20. Moreover, the relative numbers of HN, LN and NN rabbits also varies.

As control to the foregoing the effects of a number of normal sera were studied:

### *B. Effect of Normal Sera upon the Factors Determining Local Skin Reactivity.*

Normal sera from horses, goats and rabbits failed to neutralize the skin-preparatory factors in a large percentage of rabbits. It is of considerable interest, however, that some of the normal animals whose

TABLE II.

| Sera titrated | Total number of rabbits | Number showing complete neutralization; and the dilutions of the sera | | | Number showing no neutralization |
|---|---|---|---|---|---|
| | | 1:200 | 1:20 | 1:2 | |
| Normal horse serum | 17 | — | — | — | 17 |
| Normal Rabbit Serum 353 | 8 | — | — | — | 8 |
| Normal Rabbit Serum 355 | 8 | — | 1 | 2 | 5 |
| Normal Rabbit Serum 336 | 3 | — | — | — | 3 |
| Normal Goat Serum 166 | 6 | — | — | — | 6 |

In addition there were several other normal sera not included in this table which were tested in 2–3 rabbits, with no neutralization obtained.

In all of these rabbits there was a positive reaction in the control area.

serum failed to neutralize the skin-preparatory factors were subsequently injected with *B. typhosus* culture filtrate and responded with the production of neutralizing sera of high titer.

Table II shows, furthermore, that one normal serum was able to neutralize the skin-preparatory factors in low dilutions. It is seen, however, that this normal serum contained a fairly high titer of agglutinins against *B. typhosus* (Table IV). Furthermore, those sera which failed to neutralize the skin-preparatory factors contained no agglutinins or precipitins. Again, some sera which contained agglutinins failed to neutralize skin-preparatory factors.

TABLE III.

*The Effect of Heterologous Sera upon the B. typhosus Skin-Preparatory Factors.*

| Sera titrated | Total number of rabbits | Number showing complete neutralization; and the dilutions of the sera | | | Number showing no neutralization |
|---|---|---|---|---|---|
| | | 1:200 | 1:20 | 1:2 | |
| *Paratyphosus* A rabbit serum | 8 | 2 | 1 | 2 | 3 |
| *Paratyphosus* B rabbit serum | 8 | 3 | 1 | — | 4 |
| *B. coli* rabbit serum | 10 | — | — | — | 10 |
| Shiga bacillus horse serum | 10 | — | — | — | 10 |
| Flexner bacillus rabbit serum | 10 | — | — | — | 10 |
| Mt. Desert bacillus rabbit serum | 8 | — | — | — | 8 |
| *B. avicida* rabbit serum | 9 | — | — | — | 9 |
| Scarlet fever antitoxin | 8 | — | — | — | 8 |

In all of these rabbits there was a positive reaction in the control area.

TABLE IV.

*Agglutination of B. typhosus by Various Sera.*

| Sera tested | Degree of agglutination with various dilutions of sera and a constant amount of *B. typhosus* suspension | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1:4 | 1:8 | 1:16 | 1:32 | 1:64 | 1:128 | 1:256 | 1:512 | 1:1024 | 1:2028 | 1:4056 |
| Serum 446 | 4+ | 4+ | 4+ | 4+ | 4+ | 4+ | 4+ | 4+ | 4+ | 4+ | — |
| Serum 59 | 4+ | 4+ | 4+ | 4+ | 4+ | 4+ | 4+ | 4+ | 4+ | NT | NT |
| Serum 78 | 4+ | 4+ | 4+ | 4+ | 4+ | 4+ | 4+ | 3+ | 3+ | NT | NT |
| Serum 90 | 4+ | 4+ | 4+ | 4+ | 4+ | 4+ | 4+ | 4+ | 4+ | 4+ | — |
| Goat typhoid serum | 4+ | 4+ | 4+ | 4+ | 4+ | 3+ | 2+ | 1+ | — | — | — |
| *Paratyphosus* A rabbit serum | 4+ | 4+ | 4+ | 4+ | 4+ | 3+ | 2+ | 1+ | — | — | — |
| *Paratyphosus* B rabbit serum | 4+ | 4+ | 4+ | 4+ | 4+ | 4+ | 2+ | — | — | — | — |
| *B. coli* rabbit serum | 4+ | 4+ | 4+ | 4+ | 2+ | — | — | — | — | — | — |
| Shiga horse serum | — | — | — | — | — | — | — | — | — | — | — |
| Flexner rabbit serum | 4+ | 4+ | 4+ | 3+ | 2+ | — | — | — | — | — | — |
| Mt. Desert rabbit serum | 4+ | 4+ | 4+ | 2+ | 1+ | — | — | — | — | — | — |
| *Avicida* rabbit serum | 4+ | 4+ | 4+ | 4+ | 3+ | — | — | — | — | — | — |
| NRS 336 | 4+ | 4+ | 4+ | 4+ | 4+ | 4+ | 4+ | 4+ | 4+ | NT | NT |
| NGS 166 | 3+ | 4+ | 4+ | 4+ | 4+ | 4+ | 4+ | 4+ | NT | NT | NT |
| NRS 353 | 4+ | 4+ | 4+ | 4+ | 2+ | — | — | — | — | — | — |
| NRS 355 | — | — | 4+ | 4+ | 4+ | 4+ | 3+ | 2+ | — | — | — |
| Normal horse serum | 4+ | 4+ | 4+ | 4+ | — | — | — | — | — | — | — |

—, negative.

Degree of agglutination recorded in pluses.

All these facts indicate that normal sera contain no neutralizing antibodies against the skin-preparatory factors unless they also contain agglutinins against *B. typhosus*. Conversely, however, the presence of agglutinins does not necessarily indicate that the serum possesses neutralizing antibodies against the skin-preparatory factors.

TABLE V.

*Precipitation of Various Sera by B. typhosus Culture Filtrate.*

| Sera tested | Degree of precipitation with various dilutions of sera and a constant amount of $T_L$ filtrate (undiluted) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 1:2 | 1:4 | 1:8 | 1:16 | 1:32 | 1:64 | 1:128 | 1:256 | 1:512 |
| Typhoid horse serum | 4+ | 4+ | 3+ | 3+ | 2+ | 1+ | − | − | − |
| Serum 446 | 4+ | 4+ | 4+ | 3+ | 3+ | 2+ | 2+ | 1+ | − |
| Serum 59 | 2+ | 2+ | 1+ | 1+ | − | − | − | − | − |
| Serum 78 | 4+ | 4+ | 4+ | 3+ | 2+ | − | − | − | − |
| Serum 90 | 4+ | 4+ | 4+ | 2+ | 1+ | − | − | − | − |
| Goat typhoid 165 serum | 2+ | 2+ | 1+ | − | − | − | − | − | − |
| *Paratyphosus* A rabbit serum | 4+ | 4+ | 4+ | 4+ | 4+ | 3+ | 2+ | 1+ | − |
| *Paratyphosus* B rabbit serum | 4+ | 4+ | 2+ | 1+ | − | − | − | − | − |
| *Coli* rabbit serum | − | − | − | − | − | − | − | − | − |
| Shiga horse serum | − | − | 1+ | 1+ | ± | − | − | − | − |
| Flexner rabbit serum | 3+ | 2+ | 1+ | − | − | − | − | − | − |
| Mt. Desert rabbit serum | ± | ± | − | − | − | − | − | − | − |
| *Avicida* rabbit serum | − | − | − | − | − | − | − | − | − |
| NRS 336 | − | − | − | − | − | − | − | − | − |
| Normal Goat Serum 166 | 2+ | 1+ | 1+ | − | − | − | − | − | − |
| NRS 353 | − | − | − | − | − | − | − | − | − |
| NRS 355 | − | − | − | − | − | − | − | − | − |
| Normal horse serum | − | − | − | − | − | − | − | − | − |

### *C. Effect of Heterologous Sera upon the Factors Determining Local Skin Reactivity.*

A number of heterologous sera were also employed for neutralization experiments (Tables III and IV). Only sera strongly agglutinating their homologous strains were used in this part of the work.

Scarlet fever, erysipelas, *B. coli*, Shiga bacillus, Flexner bacillus, Mt. Desert bacillus and *B. avicida* sera produce no neutralization of the *B. typhosus* skin-preparatory factors (Table III), in spite of the fact that some of the sera contained agglutinins against *B. typhosus* (Table IV). The closely allied sera (Para A and B) were able to

neutralize the *B. typhosus* skin-preparatory factors in various proportions and, as would be expected, agglutinated *B. typhosus* in a fairly high titer. The rabbits employed in these experiments can also be divided into three groups, namely, those showing neutralization in highest dilutions (HN), those showing complete neutralization only in lower dilutions (LN) and those showing no neutralization (NN).

The question arises whether the *B. typhosus* skin-preparatory factors are the same as those of Para A and B, or are only closely related. This problem cannot as yet be decided.

The following scheme is tentatively proposed for the preparation of toxic filtrate and the titration of serum:

200 cc. of tryptic digest broth (2) of initial pH 7.8 are poured to a "2000 cc." Erlenmeyer flask in order to obtain a large surface area. The entire growth of one 24 hour old agar slant culture of *B. typhosus* ($T_L$)[3] is suspended in about 10 cc. of salt solution and the emulsion is added to the contents of the Erlenmeyer flask. The period of incubation is 6 days. The culture is then filtered through paper and cotton and, finally, through a Berkefeld V candle. The filtrate is tested for sterility, stored in the refrigerator and used for a period of 2–3 weeks following its preparation. No preservative is added.

For neutralization experiments 0.25 cc. of a mixture of equal parts of filtrate and saline, or of serum[4] already diluted to the desired degree, is injected into various areas of the skin of the rabbit's abdomen, by means of the technique described on page 594. The interval between the skin and intravenous injections should be exactly 24 hours. This is very important (1) to obtain uniform results. Both the size and intensity of the reactions should be recorded. A variation in the size of the reaction within small limits can be disregarded. The intensity of the reaction is an important and reliable indication of the degree of neutralization. It may be recorded in pluses. Occasionally, discolored punctiform reactions are obtained. They should be protocolled as such. There should be obtained not less than ten rabbits showing control positive reactions with the mixture of the filtrate with salt solution.

The following schematic formula has been found useful in recording the varying factors obtained in a typical serum titration: $HN_6 2000$, $LN_4 200$, $LN_1 20$, $NN_2$.

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[3] The strain of *B. typhosus* employed in this laboratory is designated $T_L$.

[4] The majority of the sera were prepared by injecting filtrates into rabbits and goats. Some sera were obtained by injecting dead and live microorganisms, for example typhoid horse serum and some heterologous sera employed in experiments described before. It seems that the presence of intact bacterial cells in the antigens injected is not essential for the production of the above described antibodies, since sera of even higher titer were obtained by means of filtrates alone. Additional experiments are necessary.

This indicates that 6 rabbits showed complete neutralization with serum diluted 1:2000, 4 rabbits showed complete neutralization with serum diluted 1:200, 1 rabbit with serum diluted 1:20 and 2 rabbits showed no neutralization. For practical purposes the highest dilution of the serum which gives complete neutralization of the *B. typhosus* skin-preparatory factors (HN titer) should be taken as the actual titer of the serum. In the example cited, 1:2000 would be considered the titer.

## SUMMARY AND CONCLUSIONS.

It is shown in this paper that homologous immune sera are able to neutralize the *B. typhosus* skin-preparatory factors. The neutralization experiments were performed on a large number of rabbits, at least ten rabbits which showed positive control reactions being used for the titration of each serum. The rabbits into which the mixtures of *B. typhosus* culture filtrates with immune sera were injected can be divided into the following categories: those showing complete neutralization in highest dilutions (HN), those showing complete neutralization only in lower dilutions (LN) and those showing no neutralization (NN). The results indicate that the potency of a given serum as measured by the method outlined above has a direct relation to the reactions obtained in these groups of rabbits. For practical purposes the highest dilution of the serum which gives complete neutralization of the *B. typhosus* skin-preparatory factors (HN titer) may be taken as the actual titer of the serum as expressed in terms of their neutralization.

The occurrence of a phenomenon suggestive of the prozone reaction is demonstrated. It also appears that the filtrates possess an antigenicity equal to that of dead and live bacteria.

The studies on normal sera bring out the fact that normal sera fail to neutralize the *B. typhosus* skin-preparatory factors unless agglutinins can be demonstrated for *B. typhosus*. No normal sera have thus far been obtained which neutralized the skin-preparatory factors yet contained no *B. typhosus* agglutinins, but there were sera which contained these agglutinins but failed to neutralize the skin-preparatory factors. Some of the normal animals whose sera failed to neutralize the skin-preparatory factors were subsequently injected with *B. typhosus* culture filtrate and responded with neutralizing sera of high titer.

Several heterologous sera were also investigated namely, scarlet fever, erysipelas, Shiga bacillus, Flexner bacillus, Mt. Desert bacillus, *B. coli* and *B. avicida*. These did not neutralize the *B. typhosus* skin-preparatory factors.

*Paratyphosus* A and B sera on the other hand produced neutralization in various proportions. And the rabbits into which the serum-filtrate mixtures were injected could also be divided according to the results obtained into the same three groups as those with *B. typhosus* sera. It is not known yet whether this neutralization is a group reaction or whether the skin-preparatory factors are identical with those of *B. typhosus*.

It would appear from these studies that a method is available for the quantitative titration of substances in the serum which neutralize the skin-preparatory factors of local skin reactivity to *B. typhosus* culture filtrates. It should be emphasized that it is possible to control the individual susceptibility of rabbits to this phenomenon. The method should permit of considerable accuracy in the quantitative titration of the neutralizing properties of a serum when a standardized procedure is developed.

Experiments are under way to determine whether the method can be applied to the preparation of therapeutic sera. Work is also in progress to determine the effect of specific antisera upon *B. typhosus* skin-reacting factors introduced by the intravenous route.

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# BLOOD CHLORIDES IN CONDITIONS ASSOCIATED WITH PNEUMONIA.

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## INTRODUCTION.

The changes which occur in the chloride concentration of the blood and in chloride elimination in the urine in pneumonia have been much studied. No attempt will be made here to review the literature completely. References may be found in the work of Hutchison (1), von Hösslin (2), Maver and Schwartz (3), and Peabody (4); more recently in the studies of Haden (5), Peters, Bulger, Eisenman, and Lee (6), and Sunderman, Austin, and Camac (7). The serum chlorides are greatly reduced and in spite of numerous investigations no agreement has been reached as to the cause therefor, nor has tissue analysis succeeded in revealing whither the lost chlorides disappear.

The fact that the blood chloride level is lowered in some other acute infectious diseases besides pneumonia has led to the suggestion that fever is in some manner responsible for the change (8, 9). Others have attributed it to the chemical changes resulting from protein destruction analogous to the chloride loss which occurs in intestinal obstruction (10), extensive burns (11), or after injections of histamine (12). The suggestion has been made that the reduction in chlorides is related to anoxemia (13).

The complexity of the situation in pneumonia with the concomitant occurrence of fever, tissue autolysis, and anoxemia makes an analysis of the problem exceedingly puzzling. For this reason we have attempted a simplification of it by experiments in animals with the hope of discovering whether changes in the environment, internal or external, would result in a diminution in the blood chlorides. Most of our experiments yielded negative results.

TABLE I.

*The Effect of Experimental Pneumococcus Infection on the Serum Chlorides of Dogs.*

| Dog No. | Date | Time | Serum chlorides per liter | Body temperature |
|---|---|---|---|---|
| | | | *mM* | °C. |
| 4 | 2/16/28 | 11:30 a.m. | 111.6 | |
| | | 11:45 a.m. inoculated by intrabronchial insufflation | | |
| | 2/16/28 | 4:35 p.m. | 110.0 | |
| | 2/17/28 | 11:00 a.m. Survived | 110.0 | |
| 5 | 2/18/28 | 10:25 a.m. | 107.3 | |
| | | 11:00 a.m. inoculated by intrabronchial insufflation | | |
| | 2/20/28 | 11:25 a.m. | 105.8 | |
| | 2/21/28 | 4:28 p.m. Survived | 113.0 | |
| 6 | 2/18/28 | 10:50 a.m. | 110.3 | |
| | | 11:30 a.m. inoculated by intrabronchial insufflation | | |
| | 2/20/28 | 11:30 a.m. | 102.6 | |
| | 2/21/28 | 4:28 p.m. Survived | 112.2 | |
| 7 | 2/23/28 | 3:25 p.m. | 114.6 | |
| | | 3:30 p.m. injected transpleurally | | |
| | 2/24/28 | 3:00 (?) p.m. | 108.6 | |
| | 2/25/28 | 11:30 a.m. | 109.5 | 39.7 |
| | 2/27/28 | Died | | |

TABLE I—*Concluded.*

| Dog No. | Date | Time | Serum chlorides per liter | Body temperature |
|---|---|---|---|---|
| | | | *mM* | °C. |
| 8 | 2/23/28 | 3:45 p.m. | 110.1 | |
| | | 3:50 p.m. injected transpleurally | | |
| | 2/24/28 | | 107.5 | 40.2 |
| | 2/25/28 | 11:30 a.m. | 103.6 | 40.1 |
| | 2/27/28 | 4:45 p.m. | 97.6 | 40.5 |
| | 2/28/28 | | 100.2 | 38.7 |
| | 2/29/28 | | 103.9 | 39.3 |
| | 3/ 6/28 | 1:30 p.m. Survived | 107.6 | 38.4 |

## EXPERIMENTAL.

### *I. Blood Chlorides in Dogs before and after Pneumococcus Infection.*

In three dogs, Nos. 4, 5, and 6, Table I, cultures of mouse-virulent pneumococci were insufflated intrabronchially according to the method of Lamar and Meltzer (14). The chlorides of the serum were analyzed by the method of Van Slyke (15) before inoculation and at intervals thereafter. None of the dogs reacted very severely to the injection and none developed a real pneumonia consolidation. In only one (No. 6) was there a chloride drop which appeared to be significant, amounting to 6.8 per cent in 48 hours.

In two dogs the pneumococcus cultures were injected transpleurally, resulting in one instance in death with a copious pleural exudate. The exudate gave a pure pneumococcus culture of the type injected. This dog (No. 7, Table I) showed a chloride drop amounting to approximately 5 per cent. The companion dog (No. 8, Table I), which survived the injection, showed a still greater reduction, reaching 11 per cent after 4 days.

It seems true, then, that a pneumococcus infection in dogs even without a pneumonic lesion may result in a lowering of blood chlorides.

*II. The Influence of Fasting.*

To control the effect of loss of appetite and consequent fasting in the infected animals, food was withheld from two normal dogs for a period of 5 days. Water was withheld only during the last 24 hours of the fasting period. The diminution in serum chloride concentration in one dog (No. 9) amounted to little more than 1 millimol per liter; from 112.5 to 111.1. The second animal (Dog. 10) showed a drop of 3.5 millimols, from 113.0 to 109.5. This does not correspond in order of magnitude to the change which may occur in pneumococcus infection.

*III. The Factor of Anoxemia.*

Five patients suffering from pneumonia, in whom arterial blood analysis demonstrated the presence of anoxemia associated with a reduced chloride concentration did not furnish evidence of a restoration of the chlorides to their normal levels when the anoxemia was relieved by placing the patients in an oxygen chamber. This led us to believe that the chloride loss was probably not related to oxygen want, as has been suggested (13). Nevertheless, to settle the point we produced a condition of acute anoxemia in a dog anesthetized by the intravenous injection of barbital-sodium.

The animal was tracheotomized and allowed to breathe, first room air, then a mixture containing 10 per cent oxygen and 90 per cent nitrogen, and finally, after breathing this mixture for 1 hour, room air was supplied instead. The arterial blood was analyzed for its oxygen and chloride content. During the hour of breathing the oxygen-poor gas mixture the per cent saturation fell from 88.8 to 59.4, whereas the chlorides fell 2 millimols. On readministering room air the per cent saturation rose to 91.4 and the chloride concentration increased only 0.7 millimol, as shown in Table II.

It can be concluded that acute anoxemia experimentally produced is not associated with any considerable alteration in the serum chloride level.

*IV. The Effect of Tissue Destruction.*

The possible relation between the presence in the body of products of tissue destruction and the reduction in serum chlorides has been mentioned. In this connection we recalled the chloride loss which occurs in association with intestinal obstruction (10), extensive skin

burns (11), and after histamine injection (12). The pneumonic process in the lung may, perhaps, represent another such process of tissue autolysis. To simulate such a reaction, we resorted to two methods; burning the skin and the production of experimental shock

TABLE II.

*The Effect of Experimental Anoxemia on the Serum Chlorides of Dogs.*

| Time | Procedure | Gas breathed | Arterial $O_2$ content | Arterial blood $O_2$ capacity | Oxygen saturation | Serum chlorides per liter |
|---|---|---|---|---|---|---|
| | | | *mM* | *mM* | *per cent* | *mM* |
| 11:29 | 1 hr. after anesthesia | Air | 9.14 | 10.77 | 84.4 | 108.3 |
| 12:09 | Tracheotomy | | | | | |
| 1:20 | | Air | 9.80 | 11.03 | 88.8 | 105.5 |
| 1:21 | | 10% $O_2$ | | | | |
| 2:21 | | 10% $O_2$ | 6.72 | 11.32 | 59.4 | 103.5 |
| 2:25 | | Room air | | | | |
| 3:20 | | Room air | 9.78 | 10.70 | 91.4 | 104.2 |

TABLE III.

*The Effect on Serum Chlorides Produced by Clamping the Abdominal Aorta.*

| Time | Procedure | Serum chlorides per liter |
|---|---|---|
| | | *mM* |
| 10:00–10:30 | Anesthetized with barbital-sodium | |
| 12:31 | | 109.5 |
| 1:17–2:24 | Laparotomy; loose ligature about abdominal aorta; abdomen closed | |
| 2:24 | | 106.5 |
| 2:30 | Aorta clamped | |
| 4:00 | Clamp released | |
| 4:15 | | 111.5 |
| 4:45 | | 106.6 |

through muscle anemia or muscle trauma (16), thinking that this might liberate a histamine-like substance and result in a chloride drop.

In one animal, Table III, under barbital anesthesia, the abdominal aorta was clamped for 1½ hours and blood was drawn for chloride analysis 15 minutes and

45 minutes after releasing the clamp. There was a slight rise in chloride concentration in the 15 minute sample, which fell again after 45 minutes to the level prevailing immediately before clamping. In another deeply anesthetized dog the leg muscles were crushed but the chloride concentration remained practically constant for 4 hours following the operation. The maximum drop was 1.1 millimols.

Davidson (11) has shown that in human beings extensive skin burns may be accompanied by a disappearance of the urinary chlorides and a marked drop in the level of the serum chlorides. In our experiments the skin of one animal under anesthesia with a large dose of barbital was extensively burned on both sides of the thorax. A little over an hour after the burn the serum chlorides had fallen 2.6 millimols. This level was maintained for the subsequent 12 hours.

*V. The Effect of Anaphylactic Shock.*

The relationship which is believed to exist between some acute infectious processes such as rheumatic fever, in which low serum chlorides may be found, and allergic states (17) led us to try the effect of anaphylactic shock on the serum chloride level.

A dog was sensitized according to the method described by Weil and Torrey (18) by the subcutaneous injection of 5 cc. of sterile horse serum. Immediately before injection a sample of venous blood was drawn for chloride analysis. The concentration was 106.1 millimols per liter. 19 days later another blood sample was drawn, which showed 104.4 millimols per liter. The dog was then given 20 cc. of horse serum intravenously. Within 2½ minutes it manifested the typical symptoms of acute anaphylaxis. 30 minutes after the injection while anaphylactic symptoms were still present a sample of blood drawn by cardiac puncture showed a slight rise in chloride concentration to 107.1 millimols. Two more specimens of blood, taken 2 hours and 5½ hours after the serum dose, showed the chloride level to be respectively 105.4 and 110 millimols per liter. Since the original level lay between 106.1 and 104.4, it cannot be said that any reduction had occurred.

*VI. The Effect of Experimental Leucocytosis.*

To see whether the low serum chloride concentration was, perhaps, related to the mobilization of the white blood corpuscles, an artificial leucocytosis was induced in a dog by the intramuscular injection of 5 gm. of sodium nucleinate dissolved in 40 cc. of distilled water.

At 11:45 a.m. the dog's leucocyte count was 10,500 per c. mm., the chloride concentration was 109.0 millimols per liter. The animal's rectal temperature was

39.3°C. At 12:30 the sodium nucleinate was injected. 2 hours later the animal looked ill, the temperature having risen to 40.4°C. 5½ hours after injection the white count had more than doubled, 26,200, and the next morning it was 22,600. At this time the temperature was 39.6°C. and the animal looked well. The chloride concentration was 108.1 millimols per liter. The next day the chloride concentration was 110.3 millimols.

Experimental leucocytosis from sodium nucleinate injection did not elicit any change in the serum chloride level.

*VII. The Influence of Fever.*

The simultaneous occurrence of low serum chlorides and an elevation of body temperature has already been referred to. It has been suggested by some investigators that the reduction in chlorides is, indeed, the result of the febrile state (18). Haldane's interesting observations on the water poisoning of miners is a related phenomenon (19). Apparently in this condition the exposure to high atmospheric temperatures results in so much loss of chloride in the sweat that chloride elimination in the urine may be entirely suspended. The great consumption of water by the thirsty miners so unbalances the ionic equilibrium in their blood that serious symptoms may result. Haldane found that the symptoms could be averted by allowing the miners to drink a ½ per cent solution of sodium chloride. It is unlikely that any such vicarious chloride loss occurs in pneumonia. Evidence points to the retention of chlorides in the tissues rather than to an increased elimination through portals other than the kidney. The suggestion that the reduction in chlorides is the result of blood dilution awaits experimental proof. Such a suggestion is, however, consistent with the hydremia which is said to occur during elevation of body temperature.

To produce fever in animals, we used two methods: the intravenous injection of methylene blue (20); and the application of the so called diathermy current to the body (21). Both result in a rapid rise of body temperature. The second method has the advantage of permitting the degree and rate of the rise to be accurately controlled. Neither method produced a drop in the serum chloride level.

A male mongrel weighing 11.2 kilos was anesthetized by the intravenous injection of barbital-sodium. When completely insensitive, the right femoral

TABLE IV.

*The Effect of Heat and Water Ingestion on Serum Chlorides and Their Relation to Serum Water Content and Chloride Output.*

| Dog No. | Procedure | Rectal temperature | Blood volume estimated from body weight | Serum chlorides | | Serum total base | | Serum water content per liter | Serum solids per liter | Urine volume output | Urine chlorides | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Per liter | Change | Per liter | Change | | | | Per liter | In volume urine excreted |
| | | °C. | cc. | *mM* | *mM* | *m.-Eq.* | *m.-Eq.* | *gm.* | *gm.* | *cc.* | *mM* | *mM* |
| 26A | | 38.2 | 1040 | 113.0 | | 158.2 | | 927.8 | 72.2 | | | |
| | Water 1 liter | | | | | | | | | | | |
| | | 38.2 | | 105.0 | −8.0 | 151.0 | −7.2 | 933.5 | 66.5 | | | |
| 26B | | 37.2 | 1040 | 112.4 | | 163.7 | | 920.9 | 79.1 | | | |
| | Water 1 liter | | | | | | | | | | | |
| | | 37.2 | | 103.2 | −9.2 | 154.0 | −9.7 | 931.9 | 68.1 | 14.5 | 10.5 | 0.15 |
| | Diathermy | 37.6 | | | | | | | | | | |
| | | 38.5 | | 101.0 | −2.2 | 145.0 | −9.0 | 931.6 | 68.4 | 86.0 | 6.5 | 0.56 |
| | Cooling | | | | | | | | | | | |
| | | 37.2 | | 102.2 | +1.2 | 149.0 | +4.0 | 931.0 | 69.0 | 132.0 | 1.8 | 0.24 |
| 22 | Sodium-veronal | 37.9 | 1700 | 108.2 | | | | | | | | |
| | Diathermy | 41.4 | | 108.2 | 0 | | | | | 71.0 | 78.3 | 5.56 |
| | Water 1 liter | | | | | | | | | | | |
| | | 42.0 | | 101.2 | −7.0 | | | | | 2.4 | 80.5 | 0.19 |
| | Water 250 cc. | | | | | | | | | | | |
| | | | | 100.5 | −0.7 | | | | | 0 | 0 | 0 |
| | Cooling | | | | | | | | | | | |
| | | 37.5 | | 98.6 | −1.9 | | | | | 0.7 | 71.5 | 0.05 |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 23 | Sodium-veronal | 38.8 | 1025 | 107.0 | | | | 916.4 | 83.6 | | | |
| | Diathermy | 41.0 | | | | | | | | | | |
| | | 41.5 | | 107.8 | +0.8 | | | 921.0 | 79.0 | 9.0 | 11.0 | 0.10 |
| | Water 390 cc. | | | | | | | | | | | |
| | Water 180 cc. | | | | | | | | | | | |
| | Water 20 cc. | 40.0 | | 107.2 | −0.6 | | | 918.4 | 81.6 | 8.3 | 19.0 | 0.15 |
| 24 | Sodium-veronal | 37.5 | 1300 | 107.8 | | | | 897.0 | 103.0 | | | |
| | Diathermy | | | | | | | | | | | |
| | | 42.0 | | 111.8 | +4.0 | | | 899.8 | 100.2 | | | |
| | Water 3 liters | | | | | | | | | | | |
| | | 41.8 | | 106.2 | −5.6 | | | 904.8 | 95.2 | | | |
| | | 41.5 | | 104.8 | −1.4 | | | 900.4 | 99.6 | | | |
| 25 | Sodium-veronal | 38.2 | 985 | 110.4 | | | | 928.6 | 71.4 | | | |
| | Water 1 liter | | | | | | | | | | | |
| | | 37.6 | | 103.2 | −7.2 | | | 937.8 | 62.2 | 21.8 | 180.8 | 3.94 |
| | Diathermy | | | | | | | | | | | |
| | | 40.2 | | 102.8 | −0.4 | | | 934.5 | 65.5 | 173.0 | 17.5 | 3.02 |
| | Water 1 liter | | | | | | | | | | | |
| | | 41.0 | | 98.6 | −4.2 | | | 939.1 | 60.9 | 14.0 | 28.3 | 0.40 |
| 27 | Sodium-veronal | 37.6 | 846 | 109.6 | | | | 926.9 | 73.1 | | | |
| | Diathermy | | | | | | | | | | | |
| | | 41.3 | | 109.4 | −0.2 | | | 922.8 | 77.2 | | | |
| | | 41.0 | | 113.2 | +3.8 | | | 920.9 | 79.1 | | | |
| | Water 846 cc. | | | | | | | | | | | |
| | | 41.0 | | 113.4 | +0.2 | | | 923.0 | 77.0 | | | |

artery and left femoral veins were cannulated. A sample of arterial blood was withdrawn for chloride analysis. This showed a concentration of 107.6 millimols per liter. A 5 per cent solution of methylene blue in distilled water was then slowly infused, 18 cc. being given in approximately 2 hours. During this interval the temperature rose from 38.4°C. to 40.3°C. Within the next 2 hours it rose another 3°C. Blood drawn at the end of the experimental period after a temperature of 40–43°C. had been maintained for over 2 hours showed no drop in chloride concentration, which remained at 108.9 millimols per liter.

Experiments of a similar character in which elevation of body temperature was produced by diathermy gave similar results. Serum was analyzed for chlorides by the method of Van Slyke (15), for total base by the method of Van Slyke, Hiller, and Berthelsen (22), and for water content by drying at 110°C. to constant weight. The results are shown in Table IV.

Dogs 22, 23, 24, and 27, Table IV, were anesthetized with barbital-sodium, the femoral artery was cannulated, and diathermy applied to the thorax. Although there was a rise in temperature in each case, varying from 1.3–4.5°C., the serum chlorides were unchanged except in No. 24, in which case they rose 4 millimols per liter. When these same animals were given large volumes of water by stomach tube, with continuation of diathermy, the serum chlorides dropped in one animal (No. 22, Table IV) as much as 7 millimols per liter in an hour. When the volume of water given was less than the equivalent of the blood volume of the animal the serum chlorides showed no significant change, as in No. 23, which showed a drop of only 0.6 millimol per liter. When water was given an hour before the diathermy, as in No. 25, the serum chlorides fell 7.2 millimols per liter as a result of the water alone. After diathermy there was a further decrease of only 0.4 millimol. After the administration of another liter of water the serum chlorides fell 4.2 millimols. The serum water content in these experiments generally showed a rise when the chlorides fell.

These experiments seem to point to the conclusion that the drop in serum chlorides is due entirely to the dilution of the serum by the water administered, and heat itself is without effect.

The effect of water ingestion on serum chlorides has been investigated by Underhill and Sallick (23). They found a drop in chlorides after ingestion of large volumes of water, equivalent to 8 times the blood volume of the dogs. They demonstrated a blood dilution by a fall in the hemoglobin. They attributed the fall in serum chlorides to increased chloride excretion.

We performed two dilution experiments.

In No. 26A, Table IV, the dog was given 1 liter of water without diathermy. In an hour the serum chlorides fell 8 millimols per liter, the serum total base fell 7.2 millimols per liter, and the water content of the serum increased in amount proportional to the decrease of chlorides and base. In No. 26B, Table IV, the same experiment was repeated with similar results, then diathermy was applied with only slight further changes in serum chlorides and water content, and the total base continued to drop, then rose slightly during the process of cooling.

The amount of chlorides eliminated by the kidneys during the experiments was in most cases less than 1 millimol, so that the drop in serum chlorides could not be accounted for by excretion. In two cases, Nos. 22 and 25, Table IV, when the excretion was greater than this, it still did not account for the change in the serum. In No. 22, when the excretion of chlorides was 5.56 millimols in an hour, the serum chlorides showed no change during diathermy, whereas when water was given the serum chlorides fell 7 millimols per liter with only 0.19 millimol excreted.

## DISCUSSION.

The object of this research was to find an experimental procedure analogous, if possible, to the one which operates spontaneously in pneumonia by which the chloride level in the dog's serum could be rapidly lowered for the purpose of studying both the mechanism and the effect of a lowered chloride level. None of the methods that were tried resulted in a chloride change which we could regard as significant. The only exceptions to this statement were the cases of experimental pneumococcus infection in dogs. In these cases two animals showed a change apparently of the same order of magnitude as is seen in the blood following the ingestion of a large volume of water. This change was due, we believe, to blood dilution, and did not appear to be influenced by the temperature of the animal. Despite the negative character of our experimental results, we present them because of the perplexity and confusion of the problem.

## SUMMARY AND CONCLUSIONS.

Attempts have been made in dogs to lower the serum chlorides by means of various procedures. Of these the withholding of food, anoxemia, tissue destruction, anaphylactic shock, leucocytosis, and fever yielded negative results. Ingestion of large amounts of water lowered the chloride level independently of body temperature. The chloride drop under these circumstances could not be accounted for by excretion and appeared to run parallel with an increase in the serum

water content. Experimental pneumococcus infection in two animals reduced the serum chloride concentration.

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# REACTIONS OF RABBITS TO NON-HEMOLYTIC STREPTOCOCCI.

## I. General Tuberculin-Like Hypersensitiveness, Allergy, or Hyperergy Following the Secondary Reaction.

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### INTRODUCTION.

In previous communications (1) we reported the phenomenon of secondary reaction to certain strains of green streptococci. Briefly this consists of an inflammatory reaction which appears about the 8th to 10th day after intracutaneous inoculation of rabbits with these microorganisms and at a time after the primary reaction has receded. Because we were unable to obtain the reaction in any species of animal except the rabbit it was thought that it might be a hypersensitive reaction of the nature of the Arthus phenomenon; but a number of experiments designed to test this hypothesis failed to prove it (2). As many features of the secondary reaction recalled certain points in the type of allergy seen in tuberculous infection attempts were made to determine whether it might not represent an allergic reaction of this type. The object of this communication is to present the detailed evidence which indicates that after the development of the secondary reaction the animals have a type of hypersensitiveness which closely resembles so called tuberculin allergy.

### *Methods.*

The tests used to prove that an animal was hypersensitive have been three: ophthalmic, cutaneous and lethal. The first two of these have proven the most useful because they could be repeatedly applied to the same animal and thus variations in its allergic state be roughly estimated.

*Ophthalmic Reaction.*—The reactivity of the eye was tested as follows: The eye was anesthetized by instilling a drop of 10 per cent cocaine into the conjunctival sac. After 5 minutes the upper quadrant of the cornea was lightly scarified with the point of a very sharp iridectomy knife so that two or three very superficial abrasions passed from the center of the cornea, upwards to within 2 or 3 mm. of the sclerocorneal junction; but care was taken not to touch the sclera. Then a drop of bacterial sediment was placed in the conjunctival sac with a pipette and the lid rubbed over the surface of the eyeball a few times. The bacterial sediment was a centrifugate of an 18-hour blood broth culture.

In a normal rabbit such an inoculation was followed by very little reaction; usually nothing could be seen except a very slight gray streak at the site of scarification. Occasionally there was slight injection of the ocular conjunctiva for a few millimeters above the sclerocorneal junction, which sometimes persisted for 48 hours, but as a rule, when present, disappeared after 1 day.

In an animal in which a secondary reaction had been induced by several fair sized intracutaneous inoculations of green streptococci an entirely different reaction to corneal inoculation often occurred. The day following inoculation there was a distinct injection of the scleral conjunctiva in the upper quadrant; this increased in intensity for 2 to 4 days, and at times was so marked that the white appearance of the sclera was practically obliterated. In marked reactions there was distinct congestion of the palpebral conjunctiva of the upper lid, and with the most intense reactions distinct edema of this conjunctiva. Simultaneously turbidity of the upper quadrant of the cornea often appeared. This at first was seen along the lines of scarification but later usually involved the whole quadrant; in very severe reactions it involved the whole upper half and even extended to the lower half of the cornea. Usually, however, when well developed it was most intense near the sclerocorneal junction on either side of the scarification, and diminished in intensity towards the center of the cornea. At times corneal turbidity did not appear for 2 or 3 days. The turbidity persisted for varying periods according to the degree of hypersensitiveness of the animal. When slight it was present for only 2 or 3 days; when marked it lasted for 10 to 15 days. Diminution in corneal turbidity was usually preceded by decrease in the conjunctival congestion. Then gradually the opacity of the cornea diminished until macroscopically only a faint scar remained at the site of instrumental traumatization. Before this occurred, however, a distinct vascular reaction was observed. A day or two after the appearance of turbidity loops of small blood vessels appeared at the upper pole of the cornea and gradually grew downwards towards its center, most marked along the line of scarification. This pannus was usually grossly visible for 1 to 3 days after corneal turbidity had diminished. If the rabbit remained hypersensitive and 1 or 2 months later the cornea was similarly scarified in another quadrant and reinoculated these loops of vessels in the upper quadrant became more quickly visible than following the first inoculation; they also appeared earlier than the pannus at the site of the second corneal inoculation. This demonstrates that although the vessels could no longer be

seen with the unaided eye they probably remained latent but ready to function when injury was inflicted on the eye later. Similar vascular latency was described by Lewis and Montgomery (3) in tuberculous infection of rabbits' eyes. Some injection of the upper portion of the iris was sometimes seen early, but if reaction of the iris was a feature of the process it was masked by the clouding of the overlaying cornea. Gross outpouring of exudative cells into the aqueous humor to the extent that it became turbid practically never occurred.

In the presence of the most severe reactions there was some mucopurulent secretion in the conjunctival sac, occasionally so abundant that it glued the lids together. Films of this exudate showed an admixture of pus cells and a few desquamated epithelial cells of conjunctival origin; but bacteria were never recognizable. Cultures of the conjunctival secretion and mucopurulent exudate uniformly failed to demonstrate viable streptococci.

These reactions resembled those severe lesions induced in tuberculous animals by the instillation of tuberculin into the conjunctival sac, and recalled some of the severe reactions seen in tuberculous patients at the time when Calmette's reaction was widely used. The interesting point is that green streptococci are so innocuous, relatively speaking, when applied in this manner to a normal rabbit's eye, yet set up such severe inflammatory reactions in the eyes of animals which have been hypersensitive. Naturally the intensity of the response varied within fairly wide limits, doubtless due to varying degrees of the general state of hypersensitiveness of the animals. Detailed evidence concerning this point will be given later. It may be stated here, however, that control hypersensitive animals tested in the same manner with plain broth never gave a positive eye reaction.

*Lethal Reaction.*—It was early noted that after a rabbit had shown a secondary reaction attempts to immunize it by intravenous injection of homologous streptococci in doses well tolerated by normal rabbits often resulted fatally. The deaths occurred between 18 and 48 hours following inoculation. Autopsy revealed a fairly constant gross alteration in certain organs: the lymph nodes were much enlarged and showed both over their surfaces and throughout their substance many hemorrhagic areas from pin-point to 1 or 2 mm. in diameter. The lymphatic tissue of the intestines was swollen and often showed hemorrhages. The thymus was enlarged from a normal of about 1 gm. to 3 or 4 gm. in weight, had a reddish gray edematous appearance and was filled with red punctate areas similar to those seen in the lymph nodes. The

marrow in the shafts of the long bones had a similar hemorrhagic appearance. While it was evident that the tissues of the hematopoietic system suffered severely in this reaction, the spleen, as a rule, did not show much gross evidence of damage. Although at times small focal hemorrhages were seen in the pericardium, endocardium and myocardium, visceral hemorrhages were rare compared with the frequency of those in the lymphatic tissues.

After the lethal test was applied more systematically it was noted that animals would sometimes appear very sick for 24 or 48 hours and then recover. If these animals were chloroformed on the 3rd or 4th day, similar, but less intense, gross pathological changes were seen. Normal rabbits and most animals that had not shown secondary reactions, on the other hand, manifested little, if any, clinical reaction to the usual test dose of streptococci, *i.e.*, the centrifugate of 4 cc. of broth culture per kilo body weight given intravenously, and if sacrificed the 3rd or 4th day by chloroforming, had little if any gross alteration of their lymphatic system. Occasionally animals which had previously failed to show secondary reactions at the site of their primary inoculations, succumbed to intravenous inoculation and showed characteristic postmortem alterations. The correlation of this lethal reaction with secondary reactions will be shown later.

*Cutaneous Reaction.*—Still another evidence of the altered reactivity of rabbits was furnished by reinoculating the skin with small doses of bacteria, first, doses that gave reactions in normal animals, and second, those that failed to elicit any gross reactions in normal rabbits. In the first instance it was observed that rabbits gave much more marked reactions at the time of second inoculation than originally, and also more intense lesions than normals simultaneously inoculated with comparable doses of the same culture. This is well brought out in the following experiment.[1]

[1] The choosing of proper rabbits and preparation of their skin for testing was carried out in the following manner.

It was necessary to select the animals carefully because certain of them had pigmented skins and also areas in which the hair was coarse. This selection was easily accomplished by grasping the rabbit with the two hands and blowing gently from the thigh forward to the shoulder. In this manner the areas of pigmented skin were noted. These latter areas were found to be covered by a coarser hair.

*Experiment 1.*—A series of rabbits were inoculated intracutaneously with the sediment of 24-hour blood broth culture of Strain V92/0/10. Group A received 4 injections of 5 cc. each, Group B, 2 of 5 cc. each and Group C, 1 of 5 cc., and

TABLE I.

*Average of Sum of Diameters of Lesions Following Reinoculation.*

| | Animals with previous secondary reaction | | | | Animals without previous secondary reaction | | | |
|---|---|---|---|---|---|---|---|---|
| | Number of animals | Amount of inoculum | | | Number of animals | Amount of inoculum | | |
| | | $10^{-1}$ cc. | $10^{-2}$ cc. | $10^{-3}$ cc. | | $10^{-1}$ cc. | $10^{-2}$ cc. | $10^{-3}$ cc. |
| Group A | 5 | 67 | 37 | 28 | 1 | 62 | 37 | 25 |
| " B | 5 | 71 | 34 | 22 | — | — | — | — |
| " C | 5 | 54 | 32 | 20 | 1 | 44 | 24 | 18 |
| Controls | 3 | 39 | 20 | 9 | — | — | — | — |

Figures indicate average sum of diameters in millimeters.

all were observed daily for secondary reactions. 16 days later all were inoculated in 3 areas with the sediment of $10^{-1}$ cc., $10^{-2}$ cc. and $10^{-3}$ cc. of homologous culture respectively. At the same time 3 normal controls were inoculated similarly.

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For the actual removal of the hair one required *impure* barium sulfide; it would not work if chemically pure. We have used Mallinckrodt's Barium Sulphide, Gray, Approx. 65 per cent. After wetting the hair thoroughly with water a fairly heavy coating of this powder was dusted on to the side of the rabbit. A heavy cotton swab on a stiff wire or stick was made wet and rubbed gently over the powdered area for about 1 to 1½ minutes; in this way a thick paste was made in the fur. As soon as it was noted that the hair was coming away the rabbit was held under running water until the paste was carefully and completely washed off by gently rubbing with the swab during the washing. If the operation had been properly performed the hair usually came away cleanly. If all the areas were not depilated the process was repeated over these areas, but it was found that there was danger of burning if the reagent was allowed to touch the bare skin. When burning resulted it was usually due to too vigorous rubbing or to allowing the paste to remain on the skin too long. It was also necessary to exercise care in removing all the barium sulfide from the hair at the margins of the denuded area. Following the washing this area was dried by gently patting with a towel and the animal placed back in the cage. It was found better to make the depilation 1 to 3 days before using the animals, for at the end of this period any burning of the skin could be detected and the areas avoided when applying the skin tests.

The maximum reaction occurred 48 hours after inoculation. Table I gives the average of the sum of the diameters of the lesions, measured at right angles to each other, recorded in the different groups at this time. One rabbit in each of Groups A and C had previously failed to show secondary reactions; and in these two the lesions resulting at the time of the second inoculation were slightly smaller than the average for their corresponding group. The figures in this table indicate clearly that the skin reactivity of the previously inoculated rabbits was from one and one-half to two times as intense as that of the controls. The animals of Groups A and B, which had received 4 and 2 lesions respectively, were more highly sensitive than those of Group C. Ophthalmic and lethal tests also demonstrated a similar high degree of sensitiveness of these two groups, compared with Group C, which in turn was more sensitive than the normal controls. In thickness and duration the lesions also showed striking differences. At the site of $10^{-3}$ cc. inoculations the most sensitive animals had indurated nodules from 2 to 2.5 mm. in height; the less sensitive animals had smaller lesions from 1 to 1.5 mm., and the controls had soft lesions 1 mm. or less. The average duration of these smallest lesions in the sensitive group was 8 days compared with 3 days in the controls. In still another respect the hypersensitive animals showed differences at the site of skin inoculation, *viz.* in color of the lesions, especially on the 2nd or 3rd day. Frequently the smaller lesions at this time had a dull red or dull pinkish red hue which did not disappear so readily on pressure as did the brighter red color seen in comparable lesions in normal animals. The color in these hypersensitive lesions reminds one somewhat of that seen in chronic granulomata such as lupus nodules or nodular syphilides.

In measuring the reactivity of the skin of rabbits with various doses of blood broth cultures of green streptococci it was observed that most normal rabbits gave no reaction with $10^{-4}$ cc. when this amount of culture was diluted with normal salt solution or plain broth and injected intracutaneously in quantities of 0.05 cc. On the other hand, animals previously injected intracutaneously with larger doses of culture showed lesions when tested later with these small quantities. Often the lesions in various animals had similar diameters, but those in the more sensitive animals were more raised, more indurated, duller red in color and persisted longer. It seemed, therefore, advisable to attempt to express the degree of reaction by the volume of the reacting macule, papule or nodule. The height of the lesion was estimated from measurements with special calipers. As the lesions were practically round, a rough estimation of their volume could be made by applying the formula $v = \frac{1}{6}\pi h(h^2 + 3a^2)$ where $v$ = volume, $h$ = height of lesion and $a = \frac{1}{2}$ of the average diameter. As a rule $a$ was estimated

as $\frac{1}{4}$ of the sum of the two longest diameters taken at right angles to one another. From curves constructed from this formula for lesions of various sizes the volumes of measured lesions could be rapidly computed. It is recognized that a large source of error is inherent in this method; first because of the amount of reaction that may occur in the deeper layers of the skin without producing a comparable thickness of the papule; second, on curved surfaces such as presented by the contour of the rabbit's body it is difficult to estimate the height of the lesion accurately; and third, because different lesions on the same animal resulting from the same sized inoculum may vary in size,

TABLE II.

*Correlation of Secondary Reactions, Ophthalmic Reactions and Test Lesions with $10^{-4}$ Cc.*

| Group | A | B | C | D | E (sick) |
|---|---|---|---|---|---|
| Secondary reactions | + | + | − | − | − |
| Ophthalmic reactions | ± to +++ | − | ± | − | − |
| Number of animals | 8 | 21 | 1 | 5 | 4 |
| Average size of $10^{-4}$ cc. lesions, *c. mm* | 109 | 61 | 215 | 47 | 12 |
| Above average | 4 | 10 | − | 2 | 3 |
| Variation in size, *c. mm* | 110–180 | 65–120 | − | 75–92 | 13–20 |
| Average | 1 | 1 | − | − | − |
| Below average | 3 | 10 | − | 3 | 1 |
| Variation in size, *c. mm* | 54–72 | 25–48 | — | 23 | 0 |

− signifies negative reaction.

± to +++ signifies strength of reaction.

either because of differences of reactivity of skin of different textures and anatomical structure, or because the inoculum is not always introduced at the same depth. But in spite of these sources of error we have found this method of estimating the degree of sensitiveness to be of distinct value. The correlation of the intensity and frequency of the three reactions and their variation among rabbits is shown in Experiment 2.

*Experiment 2.*—Each of thirty-nine rabbits was inoculated intracutaneously with the sediment of 18-hour blood broth culture of Strain V110A/0/8 in 4 areas as follows: 2 with 5 cc., 1 with $10^{-1}$ cc. and 1 with $10^{-2}$ cc., and observed daily for

secondary reactions. 9 days later each animal was given a subcutaneous focus of agar inoculated with the sediment from 5 cc. of a similar culture. 38 days after the first inoculation the skins of all were tested with $10^{-4}$ cc. of homologous culture and the eye test was applied in the usual way. The results are shown in Table II.

It will be observed that the animals with positive ophthalmic reactions gave, on the average, the largest skin reactions. While twenty-nine animals had given secondary reactions about a month previous to the time of testing the skin and eye, only nine gave positive eye tests

TABLE III.

*Correlation of Secondary Reaction, Ophthalmic Reaction and Skin Test Lesion with $10^{-4}$ Cc.*

| Group | A | B | C | D | E | F | G | H |
|---|---|---|---|---|---|---|---|---|
| Secondary reaction | + | + | ± | − | ± | + | ± | − |
| Ophthalmic reaction | + | ± | + | + | ± | − | − | − |
| Number of animals | 12 | 1 | 2 | 2 | 2 | 2 | 2 | 4 |
| Average size of $10^{-4}$ cc. lesions, *c. mm.* | 88 | 65 | 55 | 29 | 20 | 22 | 33 | 22 |
| Above average | 4 | ... | 1 | 1 | 1 | 1 | 1 | 2 |
| Variation in size, *c. mm.* | 125–227 | ... | 65 | 40 | 22 | 30 | 48 | 28–40 |
| Average | ... | ... | ... | ... | ... | ... | ... | ... |
| Below average | 8 | ... | 1 | 1 | 1 | 1 | 1 | 2 |
| Variation in size, *c. mm.* | 18–85 | ... | 45 | 18 | 18 | 15 | 18 | 10–12 |

\+ signifies distinctly positive secondary reaction.
± " doubtfully " " "
− " no secondary reaction.
... " no animals in this category.

on the 38th day. It was subsequently determined that the culture had deteriorated, which offered one possible explanation of this discrepancy. However, with the exception of the one animal in Group C, the rabbits with both positive secondary and ophthalmic reactions gave the average largest skin lesions, and those failing to give these reactions had the smallest; while those with a previously positive secondary reaction and later a negative ophthalmic reaction were midway between. The reactions in the four rabbits of Group E are noteworthy. These animals were very sick from snuffles or subcutaneous abscesses and gave

very poor reactions to all the tests. Their lesions probably represented the well known failure of cachectic individuals to react to infection in the normal manner.

The correlation of secondary reactions with ophthalmic reactions and skin reactivity to $10^{-4}$ cc. inoculation made at an earlier period than in Experiment 2 is shown in Experiment 3.

*Experiment 3.*—Twenty-seven rabbits were each inoculated intracutaneously in 4 areas with the sediment of 5 cc., 5 cc., $10^{-1}$ cc. and $10^{-4}$ cc. in 0.1 cc. amounts respectively; the inoculum was an 18-hour blood broth culture of Strain V110A/0/6 recently taken out of frozen and dried stock. None of the animals showed appreciable lesions at the site of the $10^{-4}$ cc. inoculation. On the 11th day the skin reactivity was tested with $10^{-4}$ cc. and the eye was tested with the same culture in the usual manner. The results of the three reactions are summarized in Table III.

Here again it will be seen that animals in Group A with both positive secondary and ophthalmic reactions gave on the average distinctly more voluminous skin reactions. One of this group had a lesion of only 18 c.mm., one a lesion of 40 c.mm., but in all of the others the lesions were 45 c.mm. or larger. Most of the other groups were too small to permit of valuable generalization; but among the twelve animals comprised in Groups D to H the average of the lesions was below 35 c.mm. and only one lesion as large as 48 c.mm. was found. Because these animals were to be used in other work it was not feasible to apply the lethal test.

An important point to be made from the results of both Experiments 2 and 3 is that an increased reactivity of the skin of rabbits follows previous intracutaneous inoculation whether or not a secondary reaction develops at the site of the primary inoculation. But in those animals which have recently shown a secondary reaction the reactivity of the skin to reinoculation as a rule is much greater than in those rabbits which had failed to show secondary reactions.

It is impossible to determine the degree of sensitiveness in a group of animals by applying all of the tests simultaneously, because a certain amount of time must be permitted to elapse in order to study the secondary reaction, the response to cutaneous reinoculation and the ophthalmic reaction before the lethal test can be applied. Animals vary considerably in the time at which they show a secondary reaction, and also in the degree of the reaction when it is present. All degrees of

intensity of ophthalmic reactions are also seen. A summary of the results obtained in 51 rabbits in three different experiments is shown in Table IV. All animals were sensitized with Strain V92/0/10. Six had 4 inoculations of sediment from 5 cc. each; 22 had 2 inoculations of 5 cc. each; 17 had 1 inoculation of 5 cc. and 1 of $10^{-1}$ cc.; and 6 had 1 of 5 cc. The ophthalmic test was applied on the 14th or 15th day and the lethal test on the 19th day in 17 animals, and on the 22nd day in the remainder. In this lot of animals no positive ophthalmic reactions were observed in those which had failed previously to show a secondary reaction. Among the total 42 animals comprised in Groups

TABLE IV.

*Correlation of Occurrence of Secondary Reaction, Ophthalmic Reaction and Lethal Reaction.*

| Group | A | | B | | C | |
|---|---|---|---|---|---|---|
| Secondary reaction | + | + | + | + | − | − |
| Ophthalmic reaction | + | + | − | − | − | − |
| Lethal reaction | + | − | + | − | + | − |
| Number of animals | 15 | 12 | 10 | 5 | 1 | 8 |
| Percentage in group | 55% | 45% | 66% | 33% | 11% | 89% |

+ signifies positive reaction.
− signifies negative reaction.

A and B, 25, or 60 per cent, died following intravenous inoculation, a striking contrast to the results in Group C. While a comparison of Groups A and B would indicate that rabbits with a positive ophthalmic reaction were less liable to succumb to intravenous inoculation than those without, this conclusion is probably not justified because of the differences in elapsed periods following the primary inoculation.

It is obvious that the development of a hypersensitive state as indicated by the occurrence of a secondary reaction cannot be detected unless the site of the primary injection can be observed and measured daily. The demonstration of ophthalmic hypersensitiveness and of the value of the lethal test, in animals made hypersensitive by intradermal inoculation, permitted us to apply these two tests to animals which were inoculated by various other routes. Experiments 5 and 6 give the results of such a study.

*Experiment 5.*—The animals were sensitized with the sediment of 10 cc. of 18-hour blood broth culture of Strain V92/0/11 which was concentrated by centrifugation and discarding the supernatant broth. The various sites of inoculation are indicated in Table V. In Group A the sediment of 5 cc. contained in 0.5 cc. solution was injected into each knee joint after we had assured ourselves that the needle was properly placed by first injecting and withdrawing normal salt solution. The subcutaneous injections were made over the large lumbar muscles; the intramuscular injections into these muscles. The intrapleural inoculum consisted of the sediment of 10 cc. of culture and was injected into the right pleural cavity of each animal; the intraperitoneal inoculum also consisted of the sediment of a similar amount. The paranasal sinuses were inoculated by etherizing the rabbits, boring a small hole through the thin plate of bone covering the sinuses, inserting a short needle through the trephine opening and scarifying the lining mucosa somewhat; then the sediment of 5 cc. of culture in 0.1 cc. volume was injected into the sinus on each side. The intratesticular inoculations were 0.1 cc. in volume. The vaginal inoculation was made by placing a purse string suture about the introitus, inserting a blunt needle and tightening the suture; 1 cc. of concentrated culture representing 10 cc. of growth was then injected and the needle withdrawn, and the suture tied. This suture was external to the urethra. The next day, both because the vulvæ were edematous and because the animals were unable to void, the sutures were cut and considerable amount of purulent exudate was discharged.

It was possible to measure the knees, subcutaneous lesions and testicles daily. The size and character of the lesions in the lumbar muscles could also be followed; hence in these four areas an increase in the intensity of the local reaction, about the usual time of occurrence of secondary reactions, could be estimated. But where the inoculations were made into body cavities no observations of this nature were possible.

Ophthalmic tests were applied to the right eye on the 14th day after the primary inoculation and to the left eye on the 20th day. The lethal test on the 24th consisted of 4 cc. of 18-hour blood broth culture per kilo body weight. 2 or 3 days later all animals which had not died as a result of this treatment were chloroformed and autopsied. It was thus possible to estimate roughly the sensitiveness of the tissues even though the animals had not been fatally infected.

It was evident from clinical observation that there often occurred an enlargement and more marked induration of the local inoculated areas about the 7th to 12th days, an indication that the reactivity of the tissues was undergoing an alteration at this time. Ophthalmic tests also indicated that animals in all groups were sensitized as a result of these inoculations. Twenty-one out of the thirty-six animals in the entire group gave a positive ophthalmic reaction; but comparison of

TABLE V.

*Effect of Different Modes of Inoculation—into Tissues.*

| Group | Rabbit | Site of inoculation | Secondary reaction | Ophthalmic reaction | | Lethal reaction 24th day | Thymus | Lymph nodes |
|---|---|---|---|---|---|---|---|---|
| | | | | 14th day | 20th day | | | |
| A | Q571 | Knee | ± | +±D | ++ | − | − | − |
| | Q572 | | − | + | ++ | + | +++ | +++ |
| | Q573 | | + | ++ | ++ | − | − | − |
| | Q574 | | +++ | − | − | − | − | − |
| | Q575 | | − | − | − | − | ± | − |
| B | Q580 | Subcutaneous | +++ | − | − | − | − | − |
| | Q581 | | ++ | − | − | − | ± | − |
| | Q582 | | ++ | ++D | − | − | − | − |
| | Q583 | | +++ | ++D | ++D | − | + | + |
| | Q584 | | − | − | − | − | ± | ± |
| C | Q585 | Intramuscular | − | ++ | ++ | − | − | − |
| | Q586 | | ? | − | − | − | − | − |
| | Q587 | | ++ | +D? | − | − | − | − |
| | Q588 | | − | + | ++ | − | ± | − |
| | Q589 | | ++ | ±D? | − | + | ++ | ++ |
| D | Q590 | Intraperi- | 0 | ± | − | − | − | − |
| | Q591 | toneal | 0 | +± | − | − | − | − |
| | Q592 | | 0 | − | − | − | − | − |
| | Q593 | | 0 | + | + | + | +++ | ++ |
| | Q594 | | 0 | ± | − | − | ± | − |
| E | Q595 | Intrapleural | 0 | − | − | − | − | − |
| | Q596 | | 0 | + | ± | − | − | − |
| | Q597 | | 0 | ++ | ± | − | + | − |
| | Q598 | | 0 | − | − | − | ± | − |
| | Q599 | | 0 | +± | − | − | ± | − |
| | Q600 | | 0 | − | − | − | ± | − |
| F | Q601 | Paranasal | 0 | + | + | − | + | − |
| | Q602 | sinus | 0 | − | +± | − | + | − |
| | Q603 | | 0 | − | − | − | ± | − |
| | Q604 | | 0 | + | +± | − | + | − |
| G | Q605 | Intratesticular | ? | ++± | − | − | + | − |
| | Q606 | | − | − | − | − | − | − |
| | Q607 | | − | − | − | − | − | − |
| | Q608 | | + | − | − | − | − | − |
| H | Q577 | Vaginal | 0 | − | − | − | − | − |
| | Q578 | | 0 | ++ | + | − | − | − |

± to +++ signifies various degrees of intensity of reaction.
− signifies negative reaction.
0 " no observation.
D " reactions delayed in time of appearance.

the corneal sensitiveness on the 14th and 20th days showed that at the time of first testing twenty of the animals showed positive reactions contrasted with only thirteen at the second testing; and in addition a number of the reactions at the time of later testing were distinctly weaker than on the 14th day. This indicates that in general the degree of sensitiveness was decreasing. It was previously reported by us (1) that the green streptococci are rapidly killed off in the intracutaneous foci. In this experiment one subcutaneous focus excised on the 11th day was sterile; and all foci cultured at autopsy failed to show living organisms. It thus seems probable that the stimulus to sensitization, which probably occurs as a result of reaction between tissues and bacteria, is not continuous because a new supply of bacteria is not available for its production. Likewise a diminution in the degree of hypersensitiveness probably explains the small number of lethal reactions, for this test was not applied until the 24th day after the primary inoculations.

A study of the various groups reveals some results of further interest. With the ophthalmic reaction as a guide, Group G with intratesticular inoculations and Group B, subcutaneously inoculated, showed the least sensitization, for in both the eye reactions were much delayed, and occurred relatively infrequently.

While a high proportion of the animals in Groups D and E, inoculated intraperitoneally and intrapleurally respectively, showed positive eye reactions on the 14th day, they were weak or delayed in three, and in all except Rabbit Q593 had become much weaker or negative when tested on the 20th day. This animal succumbed to intravenous inoculation 4 days later, an indication that its hypersensitiveness was continuing on a high level.

Group C, with intramuscular foci, had two members in which the eye reaction was weak and delayed on the 14th day, but also had two others in which the corneal sensitiveness persisted and was as strong or stronger on the 20th day. It is noteworthy that in only two other groups was this the case. In both the intraarticularly inoculated animals, Group A, and in those with paranasal sinus inoculations, Group F, the degree of corneal sensitization persisted and was as strong or stronger by the 20th day. It is interesting to note that infection of the mucosa of the paranasal sinuses without gross abscess formation,

as revealed post mortem, should lead to such rapid and continuing hypersensitiveness. A similar persisting state from intraarticular inoculation, is also interesting in view of the fact that in only one of these animals, A574, was there a frank abscess in the joint. In all others the synovia had a gelatinous appearance.

In the one rabbit with vaginal inoculation showing positive ophthalmic reaction there was an abscess in the vaginal wall.

TABLE VI.

*Effect of Different Modes of Inoculation—Intravenous.*

| Group | Rabbit | Intravenous inoculation | Ophthalmic reaction | | Lethal reaction 19th day | Thymus | Lymph nodes |
|---|---|---|---|---|---|---|---|
| | | | 13th day | 16th day | | | |
| | | *cc.* | | | | | |
| A | Q388 | 10 | × | – | – | – | – |
| | Q389 | 10 | × | – | – | – | – |
| | Q390 | 10 | × | – | – | – | – |
| B | Q382 | 10 | – | – | – | – | – |
| | Q383 | 10 | – | ±? | – | – | – |
| | Q384 | 10 | – | – | – | – | – |
| | | | | | 10th day | | |
| C | Q320 | 5, 10 | × | × | – | – | – |
| | Q321 | 5, 10 | × | × | | – | – |
| | Q322 | 5, 10 | × | × | – | – | – |

– signifies negative reaction.
× " not inoculated at this time.

If now we contrast these results with those in a series of rabbits inoculated only intravenously, as in Experiment 6, the differences are most striking. The mode of inoculations and results are summarized in Table VI.

*Experiment 6.*—The animals of Groups A and B were inoculated intravenously with the sediment of 10 cc. of 20-hour blood broth culture of Strain V92/0/10; those of Group C with sediment of 5 cc. As we had occasionally observed deaths following early intravenous inoculation of intracutaneously sensitized animals the rabbits in Group C were reinoculated 3 days later with sediment of 10 cc. of culture. 2 days later Q321 died; but post mortem was found to have a severe *B. lepisepticum* infection; no gross lesions, however, were found in the thymus,

lymph nodes or bone marrow. The other two rabbits of Group C were again inoculated intravenously on the 10th day with sediment of 7.5 cc. of culture (4 cc. per kilo body weight) without any untoward symptoms, and with negative postmortem findings 3 days later.

The right eyes of Group B animals tested on the 13th day all failed to react. The left eyes of all surviving animals were tested on the 16th day with negative reactions in each instance except one where there was slight corneal turbidity, lasting only 2 days. Reactions of this degree sometimes occur in normal animals; hence it is safe to conclude that there was no corneal sensitization in any of the animals. All were inoculated intravenously with sediment of 10 cc. of homologous culture on the 19th day without showing any evidence of sickness afterwards. 3 days later, at autopsy, none showed any of the characteristic changes in the hematopoietic organs; Q390 showed a mild arthritis of the left knee and right shoulder. Thus lethal tests on the 4th, 10th and 19th days and ophthalmic tests on the 13th and 16th days failed to reveal any evidence of hypersensitiveness in animals in which the first inoculation was by the intravenous route and was of the same size as induced hypersensitiveness in all groups of animals in Experiment 5. Controls of the cultures used to make these tests proved that they were active in hypersensitive animals. Similar results following primary intravenous inoculations have been obtained with other strains of non-hemolytic streptococci.

It seems, therefore, that the condition necessary for the production of an early hypersensitive state, such as occurs in rabbits with the appearance of secondary reactions after intracutaneous inoculation with suitable non-hemolytic streptococci, is the production of an inflammatory focus some place in the body. Altered reaction doubtless occurs in an animal regardless of the microorganism introduced or the route through which it is introduced. There are, moreover, wide variations in the capacity of different animals to react to inoculation into similar tissues, and also distinct differences in different strains in their capacity to sensitize a group of animals. This has been already mentioned in our earlier publications, but the importance of recognizing variation in both the power of the microorganism to sensitize and the capacity of the animal to react is more clearly demonstrated in another place (4). The present discussion has rather to do with the influence of primary reaction in various tissues. When non-hemolytic streptococci of low virulence are introduced into the blood stream of normal animals they rapidly disappear and, in the doses here used, do not, as a rule, set up gross focal lesions in the tissues of the body. Their influence is probably exerted over a relatively wide area as

represented by the ramifications of the circulatory system. Doubtless certain organs bear the brunt of the attack more than others, but all are usually able to dispose of these streptococci without undergoing severe damage. When, on the other hand, these microorganisms are introduced directly into the tissues there is a local reaction, with a certain amount of tissue destruction, and before the body has been able to dispose of them effectively the inflammatory material has been produced either in sufficient quantity or of a quality to alter the bodily response in the direction of overreaction to a second inoculation at a distant site. It is interesting to note that such relatively slight reactions in the paranasal sinuses should lead to more marked sensitization than followed more marked reactions in the testicles, and also should be followed by corneal sensitization of longer duration than occurred after intrapleural or intraperitoneal inoculation. In view of the suspected rôle of streptococci in chronic arthritis the fact that general sensitization can be easily effected from intraarticular inoculation is also noteworthy.

## DISCUSSION.

From the experiments presented in this and other communications it is obvious that the production of a lesion in rabbits by inoculation with non-hemolytic streptococci into some tissue is followed by a state of hypersensitiveness. This state is made evident by increased reactivity of the skin to reinoculation with small doses of the streptococci, by the marked reactivity of the scarified cornea—a non-vascular tissue —to instillation of the streptococci into the conjunctival sac and by the death of many of the animals following intraveneous inoculation with cultures in amounts well tolerated by normal rabbits. The lesions found in the lymphatic and hematopoietic organs of these animals are grossly very similar to those described originally by Koch (5) in tuberculous animals following inoculations with large doses of tuberculin. We have observed similar lesions in normal rabbits following intravenous injections of more virulent hemolytic streptococci, and have noted ophthalmic reactions similar to those seen in hypersensitive rabbits following primary inoculation of the cornea of normal rabbits with living hemolytic streptococci. Thus, the condition of the hypersensitive rabbit has been altered in such a manner that the relatively

avirulent non-hemolytic streptococci set up immediate reactions grossly comparable to those which follow infection of normal animals with virulent hemolytic streptococci.

The death of these animals after intravenous inoculation does not occur immediately or within a period of 1 or 2 hours, nor is it accompanied by symptoms of acute shock such as were originally described by Arthus (6) in rabbits sensitized to various coagulable proteins. Neither did Auer (7) or Opie (8) describe lesions of this type in rabbits fatally shocked with horse serum or egg white. In a few animals intracutaneously inoculated with egg albumin we have not observed corneal hypersensitiveness comparable with that of the streptococcus-hypersensitive rabbits; nor have we found in the literature a description of this type of eye sensitiveness to non-bacterial coagulable proteins.

It has already been shown (2) that the secondary reaction bears no definite relationship to the presence of antibacterial immune bodies in the sera of the infected rabbits. In this respect the hypersensitiveness to non-hemolytic streptococci differs from that of rabbits immunized against non-bacterial coagulable proteins, where regardless of method of immunization a heightened antibody content of the serum is accompanied by an increased intensity of the Arthus phenomenon.

In all respects, therefore, where analogies can be applied, it seems that the hypersensitiveness of rabbits following the production of lesions with certain non-hemolytic streptococci, is comparable to that form described as tuberculin type of allergy or the hypersensitiveness of infection (Coca (9)). Zinsser (10) has clearly indicated how the two types of hypersensitiveness, or allergy, differ with respect to the presence of immune bodies; and later he expressed the opinion that the bacterial sensitization resulted from a peculiar form of autolysis of bacteria which probably occurs most readily in inflammatory foci. Roessle (11) also directed attention to the differences between the tissue reactions and incitants in the two types of hypersensitiveness.

It is, furthermore, perfectly evident that this type of hypersensitiveness or allergy is not peculair to non-hemolytic streptococci, for in such chronic diseases as tuberculosis, syphilis, glanders, blastomycosis and trichophytosis, to mention only a few, skin reactions to the respective bacterial extracts are well known. Studies in typhoid reactions by

Gay (12) and his coworkers, and in reactivity to pneumococcus extracts or nucleoproteins after pneumococcus infections, by Mackenzie and Woo (13), and by Zinsser and Grinnell (14), show that acute infections also induce a similar type of bacterial allergy. Hanger's (15) studies of the reactions of rabbits to *B. lepisepticum* filtrates also indicate that rabbits spontaneously infected with these microorganisms develop a peculiar type of allergy towards them.

In the field of streptococcus infections, especially as represented by recent developments in the study of scarlet fever and erysipelas, the accumulating evidence all indicates that bacterial allergy plays an important rôle in certain phases, at least, of these diseases. This work summarized by Zinsser (16) is well illustrated by the studies of Dochez and Stevens (17), Birkhaug (18), Zinsser and Grinnell (19) and Mackie and McLachlan (20); Kirchner (21) showed moreover that there was increasing hypersensitiveness of such an avascular tissue as the cornea to the so called scarlatinal toxin.

But it should be pointed out that in these studies although the primary inoculation in many instances was with cultures of the respective streptococci, in most instances the substance applied in testing was a broth filtrate or some bacterial fraction. In most of our studies we have used whole living non-hemolytic streptococci, though in a few instances we have tested hypersensitiveness with nucleoproteins, and filtrates. Mackenzie and Woo used nucleoproteins, and Zinsser and Grinnell used *Streptococcus scarlatinæ* nucleoproteins as well as filtrates as testing substances. Still more recently Julianelle and Avery (22) have carried out more extensive series of experiments with pneumococcus infections in rabbits with results very similar to those we have obtained with non-hemolytic streptococci.

Because of the relatively low primary virulence of non-hemolytic streptococci we have thought it best to study the type of reaction following both primary inoculation and reinoculation with living microorganisms. The low invasive capacity of these streptococci and the rapidity with which they are annihilated in the rabbit's body made it especially desirable to determine just how the animal reacted towards the whole living microorganism during the various phases of immunity and resistance. While tubercle bacilli, *Spirochæta pallida* or the various fungi found in trichophytosis have a comparable low grade of

primary virulence, the fact that they remain viable in the body of an animal and hence give rise to new foci for long periods creates a different set of conditions than follows inoculation with non-hemolytic streptococci. Primary inoculation with such microorganisms as capsulated pneumococci, hemolytic streptococci or staphylococci which have a higher invasive capacity, greater virulence and the ability to elaborate more markedly toxic substances, gives still other and more complicated conditions. Dold (23), though aware of the phenomenon of secondary reaction, did not record its occurrence in rabbits inoculated intracutaneously with a large variety of common pathogenic bacteria. Hence it appears that with this reaction of rabbits towards non-hemolytic streptococci we have available a set of factors quite favorable for analysis of certain phases of allergy and immunity.

The evidence brought out in Experiments 5 and 6 that foci some place in the body are necessary for the development of this type of allergy is most noteworthy. It is not merely a question of introducing the bacteria into the body but the fact that focal lesions are set up that seems to be important, for hypersensitiveness did not follow intravenous inoculation. Rapid destruction of the microorganisms without production of large focal lesions probably offers a suitable explanation of this phenomenon. If staphylococci, hemolytic streptococci or most strains of pneumococci, had been injected intravenously, either marked purulent focal lesions would have resulted, or the animal would have died before the allergic state could have been detected. Following intravenous injection with living tubercle bacilli there would have been produced multiple tubercles, foci of sufficient intensity to induce a condition of tuberculin allergy, and the persistence of tubercles would favor a continuance of the allergic state. In this connection, however, it is interesting to note that Petroff (24) observed that it was possible to induce a state of tuberculin allergy following inoculation of animals with killed tubercle bacilli by practically every route except intravenously. It is possible that the mode and duration of action of killed tubercle bacilli in respect of the inducing of allergy are much the same as those of non-hemolytic streptococci. The description by Calmette and Guérin (25) of secondary reactions at the site of inoculation of calves with attenuated tubercle bacilli B C G furnishes a striking parallel to our observations in rabbits. Wallgren (26), moreover, has very recently

reported the occurrence of secondary reactions in young children at the site of inoculation with Calmette's B C G bacilli, and found that cutaneous allergy to tuberculin only occurred in those children who had developed these secondary reactions.

We, therefore, seem to be in a position to give a reasonable explanation of the secondary reaction. Following the intracutaneous inoculation of rabbits with any strain of streptococcus there develops a state of tuberculin-like hypersensitiveness; but certain strains possess the capacity of stimulating the hypersensitiveness to a higher level than others, and certain rabbits are more capable of reacting, as is made evident by retesting the animals in different ways. Following the primary intradermal reaction there persists at these primary foci a certain amount of residual antigenic substance. When a sufficiently high degree of general hypersensitiveness develops the cells in the immediate vicinity of the primary lesion are in a condition to react with small amounts of suitable bacterial substances whether they are either freshly introduced or residual. The secondary reaction is, therefore, apparently an evidence of this reaction with some residual antigen persisting at the site of primary inoculation in an animal which has developed a general state of hypersensitiveness. The peculiar feature is that some strains should possess these stimulating or reacting substances to such a degree, while others are strains apparently lacking in them.

In the studies of Dochez and Stevens and of Birkhaug concerning the two phases of sensitization of rabbits towards toxic filtrates various routes of inoculation were employed. The first observers injected toxic filtrates intracutaneously, and later intravenously; the last experimenter injected living indifferent streptococci intraperitoneally. While Dochez and Stevens observed that the second phase of allergy followed prolonged intravenous inoculation, Birkhaug reported that skin desensitization followed intravenous injection of toxic filtrates. Therefore, there appears to be some contradictory evidence concerning the results following different routes of inoculation. But it should be noted that different types of streptococci were used by these two sets of workers. Nevertheless the influence of the types of lesions resulting from the different routes of inoculation has in general not been as carefully considered as it probably deserves. If our observations

concerning the effect of production of focal lesions compared with that of intravenous inoculation without focal lesions are substantiated with other bacteria, it may be necessary to revaluate certain of the factors in the production of tuberculin-like bacterial allergy.

## CONCLUSIONS.

1. Accompanying and following the evolution of a secondary reaction in the skin of rabbits after inoculation with suitable doses of certain non-hemolytic streptococci there quickly develops a general state of hypersensitiveness or allergy towards these streptococci.

2. This state is made evident by ophthalmic reactions following corneal inoculations, by much increased reactivity of the skin following intracutaneous reinoculations, and by lethal reactions, resembling tuberculin shock, following intravenous inoculations.

3. In a given hypersensitive rabbit there is a rough parallelism in the intensities of these different kinds of reactions.

4. This type of hypersensitiveness or bacterial allergy does not follow primary intravenous inoculation of rabbits with comparable doses of the streptococci employed.

5. As the development of this type of hypersensitiveness or bacterial allergy seems to accompany the production of focal lesions of a certain intensity, it is probable that in these foci are produced the substances or conditions which lead to this type of bacterial allergy.

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# STUDIES ON INDIFFERENT STREPTOCOCCI.

## III. The Allergizing Capacity of Different Strains.

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The discovery in this laboratory (1) of the secondary reaction which occurs at a definite interval following the intradermal inoculation into rabbits of properly chosen doses of certain strains of *Streptococcus viridans* has pointed the way for a number of investigations into the general field of bacterial allergy, with reference particularly to the streptococcus group (2). The results of these investigations, in conjunction with a number of findings in patients with rheumatic fever, have given rise to a working theory in which the phenomena of the disease are interpreted in terms of allergy or hyperergy to streptococci (3).[1] Most of the experimental data reported have been obtained with certain strains of *Streptococcus viridans*. In view, however, of the fact that the indifferent streptococci have been considered to play a part in the production of the disease (4), and in some quarters have been assigned an exclusive etiologic rôle, it has seemed desirable to investigate these organisms with a view to determining their potentialities as allergizing agents. A number of interesting observations along this line have been published recently by Birkhaug (5). His animals were inoculated intraperitoneally and were tested only with filtrates.

Inasmuch as in the case of *Streptococcus viridans* the capacity to produce the secondary reaction in rabbits had served as a fairly reliable index of the allergizing activity of given strains, the obvious approach to the problem was to observe the frequency with which this reaction occurred following the properly executed inoculation with various strains of indifferent streptococci. For this purpose a number of

[1] A similar theory was quite independently developed by Zinsser, *Bull. New York Acad. Med.*, 1928, iv, 351, and previously suggested in an article by him and Grinnell, *J. Immunol.*, 1925, x, 725.

cultures were selected from among those carried in stock at the time. Twenty-four examples of Type I and twenty of Group X (6) were studied; among the latter were included eleven which in our hands failed to ferment inulin. Some of the strains had been isolated from patients suffering from active rheumatic fever; others from persons in sound health who had never suffered from the disease; still others from hospital patients suffering from conditions unrelated to rheumatism.

TABLE I.

*Scheme for Inoculation of Rabbits and Tabulation of Results.*

| Rabbit No. | | | |
|---|---|---|---|
| 1 | A | B | C |
| 2 | B | C | D |
| 3 | C | D | E |
| 4 | D | E | F |
| 5 | E | F | A |
| 6 | F | A | B |

## *Methods.*

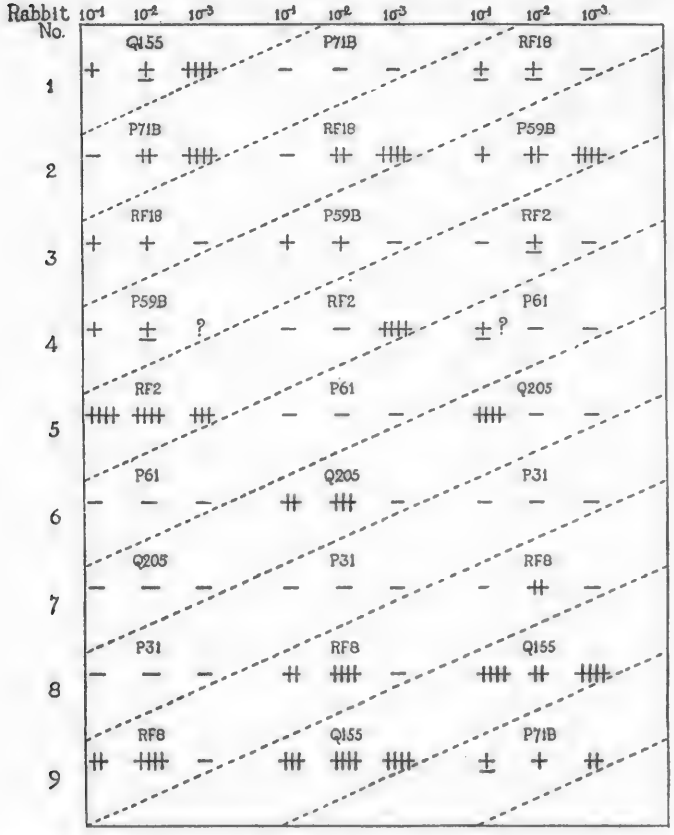
Inasmuch as only in the rabbit has the gross secondary reaction been observed, this was of necessity the experimental animal chosen. Inoculations were made into areas of unpigmented skin depilated at least 24 hours previously with a watery paste of commercial barium sulfide (Mallinckrodt). The bacteria were grown for 24 hours in phosphate broth, pH 7.8, containing 2 per cent of defibrinated rabbits' blood and 0.05 per cent of dextrose. When such cultures were administered in amounts less than $10^{-1}$ cc., dilutions were made in phosphate broth such that the dose desired was contained in a volume of 0.05 cc. As a rule each strain was administered in three doses, $10^{-1}$ cc., $10^{-2}$ cc. and $10^{-3}$ cc.; but in a few experiments the $10^{-1}$ cc. dose was omitted. Each rabbit was tested with three strains in the dosages given and each strain was tested in at least three animals.

By a method of staggering the inocula it was arranged that no two animals received the same three strains. Results were tabulated in accordance with this scheme, which is illustrated in Table I. Thus six strains, which might be designated A-F, would be injected into six rabbits, as indicated in Table I. Certain advantages accrue to this method of study, for both variation in reactivity of the rabbits and in allergizing capacity of the microorganisms are demonstrated. In the first place, a small number of animals are incompetent to develop the secondary reaction, whether through illness (cachectic response), or because of the existence of a special condition of natural immunity. On the other hand, many strains of indifferent streptococci, more particularly of Group X, are endowed with little or no allergizing capacity. The percentage of rabbits refractory to sensitization is so small that it is unlikely that more than one in four or five will be encountered in any single group. By reading horizontally it is a simple matter to determine the

presence of refractory animals, as well as of those unusually responsive to sensitization. By reading along the dotted diagonals the number and degree of the secondary reactions encountered will serve as a fair index to the sensitizing power

TABLE II.

*Variations in Allergizing Capacity of Nine Strains of Indifferent Streptococci Tested in Nine Rabbits.*




of the strains. The method is, of course, of less value when one is dealing with a number of strains, few or none of which possess more than a weak or irregular allergizing capacity. Under such circumstances it is desirable to introduce strains

of known potentialities as controls; in Table I Strains A and D might be thus chosen; the only alternative to this, in case of the development of a small percentage of secondary reactions, is repetition of the experiment.

The results of one among several experiments are shown in Table II. Nine rabbits were each inoculated in nine areas with doses and strains of different streptococci as indicated in the headings. All of the lesions were measured daily for 3 weeks. The scheme used in charting the intensity of the secondary reactions was as follows: the two diameters of the lesions were added, and from this result was subtracted the sum of the two diameters observed when the lesion was at its minimum preceding the development of the secondary reaction. When this difference amounted to 1–3 mm., the ± sign was charted; 4–6 mm., +; 7–9 mm., ++; 10–12 mm., +++; 13 mm. or more, ++++. The − sign indicates absence of secondary reaction.

TABLE III.

*Intensity of Secondary Reactions from Type I Strains.*

| Total secondary reactions | Intensity | | | | |
|---|---|---|---|---|---|
| | ± | + | ++ | +++ | ++++ |
| 150 | 16<br>10.7% | 26<br>17.3% | 24<br>16.0% | 13<br>8.7% | 71<br>47.3% |

For meaning of + and − signs, see text.

In this experiment Rabbits 1, 3, 4 and 7 were poorly adapted to the purpose in hand, as they were obviously in varying degree refractory to sensitization, No. 7 almost completely so. This unusually high proportion of unsatisfactory rabbits might have invalidated the experiment had it not been for the peculiar distribution of the strains. However, by reading along the diagonal lines and making allowances for the refractory rabbits as their levels are reached, it is made clear that Strains Q155, P71B, RF18, P59B, RF2, Q205 and RF8 really possess the allergizing power, while Strains P31 and P61 are devoid of it, for in areas inoculated with the latter microorganisms secondary reactions failed to appear even though the tests were made on such highly reactive animals as No. 8 and No. 5 respectively. Variations among the positively reacting microorganisms in capacity to stimulate secondary reactions were also made evident in this experiment. For example, Strain Q155 gave good, even though not marked, reactions in such a poorly reacting animal as No. 1; while Strain RF18 gave very poor reactions in this animal and in No. 3, but better reactions in the more reactive No. 2.

Ophthalmic tests were carried out as described elsewhere (3), by application of culture sediment to corneas previously scarified under novocaine anesthesia.

RESULTS.

With the 24 strains of Type I, 215 lesions were produced in 82 animals. In 150 (69.7 per cent) of these lesions secondary reactions appeared within 3 weeks following the date of inoculation, and usually before the 12th day.

In Table III the 150 secondary reactions have been analyzed with respect to their intensity, in accordance with the arbitrary formula

TABLE IV.

*Incidence of Secondary Reactions in Lesions Resulting from Different Inocula of Type I Strains.*

| | Size of inocula | | |
|---|---|---|---|
| | $10^{-1}$ cc. | $10^{-2}$ cc. | $10^{-3}$ cc. |
| Total lesions | 51 | 82 | 82 |
| Secondary reactions | 37 (72.6%) | 69 (84.2%) | 44 (53.7%) |

TABLE V.

*Intensity Distribution of Secondary Reactions among Different Inocula of Type I Strains.*

| | | Size of inocula | | |
|---|---|---|---|---|
| | | $10^{-1}$ cc. | $10^{-2}$ cc. | $10^{-3}$ cc. |
| Total secondary reactions | | 37 | 69 | 44 |
| Intensity | ± | 4 (10.8%) | 11 (15.9%) | 1 (2.3%) |
| | + | 8 (21.6%) | 15 (21.7%) | 3 (6.8%) |
| | ++ | 12 (32.4%) | 11 (15.9%) | 1 (2.3%) |
| | +++ | 2 (5.4%) | 6 (8.7%) | 5 (11.4%) |
| | ++++ | 11 (29.7%) | 26 (37.7%) | 34 (75.2%) |

mentioned above. Nearly 50 per cent of the reactions were of sufficient degree to be expressed as ++++. In Table IV the 215 lesions produced by the Type I strains have been analyzed with respect to the occurrence of the secondary reaction in the lesions resulting from the various inocula. The high percentage (53.7 per cent) of secondary reactions in the $10^{-3}$ cc. lesions is noteworthy, and is in contrast to observations made with *Streptococcus viridans*; for we have repeatedly observed that secondary reactions in lesions resulting from inocula-

tion with $10^{-3}$ cc. of *Streptococcus viridans* cultures are relatively uncommon.

In Table V are analyzed the lesions resulting in secondary reactions with respect to their distribution by intensity among the three inocula. The tendency for the $10^{-3}$ cc. lesions to show more strongly marked reactions than the $10^{-1}$ cc. lesions is largely attributable to the method

TABLE VI.

*Intensity of Secondary Reactions from Group X Strains.*

| | Total secondary reactions | Intensity | | | | |
|---|---|---|---|---|---|---|
| | | ± | + | ++ | +++ | ++++ |
| Inulin fermenters | 41 | 9<br>21.9% | 20<br>48.8% | 6<br>14.6% | 1<br>2.4% | 5<br>12.2% |
| Non-fermenters | 39 | 9<br>23.1% | 11<br>28.2% | 7<br>17.9% | 0<br>— | 12<br>30.8% |

TABLE VII.

*Relation between Size of Inoculum and Incidence of Secondary Reaction in Group X Lesions.*

| | | Size of inocula | | |
|---|---|---|---|---|
| | | $10^{-1}$ cc. | $10^{-2}$ cc. | $10^{-3}$ cc. |
| Inulin fermenters | Total lesions | 23 | 23 | 23 |
| | Secondary reactions | 17 (74.0%) | 16 (69.6%) | 8 (34.8%) |
| Non-fermenters | Total lesions | 25 | 34 | 34 |
| | Secondary reactions | 15 (60.0%) | 16 (47.1%) | 8 (23.5%) |

of recording by diameters. The lesions produced by $10^{-1}$ cc. doses of Type I strains are relatively quite large. In the case of such large lesions the secondary reaction manifests itself as much by increase in volume as by lengthening of diameters, whereas smaller lesions frequently increase disproportionately in circumference. For this reason the data of Table IV are perhaps a truer picture of conditions than are those of Table V.

The twenty strains of Group X have been analyzed in two groups according to their capacity to ferment inulin in Hiss serum water tubes. With nine strains of inulin-fermenting organisms 69 lesions were produced in 23 animals. In 41 (59.4 per cent) of these lesions secondary reactions appeared within 2 weeks of the date of inoculation. Similarly, with the eleven non-fermenting strains 93 lesions were produced in 34 animals; in 39 (42 per cent) of these lesions secondary reactions appeared within the same period of time.

TABLE VIII.

*Intensity Distribution of Secondary Reactions among Different Inocula of Group X Strains.*

| | | | Size of inocula | | |
|---|---|---|---|---|---|
| | | | $10^{-1}$ cc. | $10^{-2}$ cc. | $10^{-3}$ cc. |
| Total secondary reactions | | | 17 | 16 | 8 |
| Inulin fermenters | Intensity | ± | 4 (23.5%) | 4 (25.0%) | 1 (12.5%) |
| | | + | 11 (64.7%) | 7 (43.7%) | 2 (25.0%) |
| | | ++ | 2 (11.8%) | 3 (18.7%) | 1 (12.5%) |
| | | +++ | 0 — | 1 (6.3%) | 0 — |
| | | ++++ | 0 — | 1 (6.3%) | 4 (50.0%) |
| Total secondary reactions | | | 15 | 16 | 18 |
| Non-fermenters | Intensity | ± | 4 (26.7%) | 3 (18.7%) | 2 (25.0%) |
| | | + | 4 (26.7%) | 7 (43.7%) | 0 — |
| | | ++ | 2 (13.3%) | 2 (25.0%) | 3 (37.5%) |
| | | +++ | 0 — | 0 — | 0 — |
| | | ++++ | 5 (33.3%) | 4 (50.0%) | 3 (37.5%) |

In Table VI these reactions are analyzed with respect to their intensity. Not only does the secondary reaction appear with less frequency in the lesions given by Group X strains, but comparison with Table III shows that there is a decided tendency for it to be less intense when present.

In Table VII the total Group X lesions have been analyzed with respect to the relation between size of inoculum and incidence of the secondary reaction. Whereas the incidence of the secondary reaction in the $10^{-1}$ cc. lesions approaches that observed for the Type I strains, there is a decided tendency for it to be lower in the cases of the smaller lesions. This is obvious from comparison of Table VII with Table IV.

In Table VIII is given the intensity distribution of the secondary reactions among the lesions resulting from the different inocula of Group X strains.

## DISCUSSION.

Even a cursory examination of Tables III to VIII will reveal the fact that the capacity to give rise to lesions in which a secondary reaction will appear is a fairly constant characteristic of the indifferent streptococci. In the case of *Streptococcus viridans* only strains derived from patients suffering from active disease of some sort were found to possess this capacity, while strains saprophytic in the respiratory passages were devoid of it (1), but this point was not investigated so extensively as in the present study. As regards the indifferent streptococci, however, the source of the strains has been immaterial; certain cultures isolated from the throats of normal individuals have given rise to secondary reactions as frequently and in as great intensity as have any of those derived from patients with manifestations of active rheumatic fever.

The capacity to yield the secondary reaction is greater for the strains of Type I than for those of Group X; and within the latter group the inulin-fermenting organisms show it more frequently than do those which fail to ferment this carbohydrate. In the case of the latter organisms it is found that the $10^{-1}$ cc. lesions do not yield the secondary reaction any more frequently than do the $10^{-3}$ cc. lesions of the Type I strains. Furthermore, examination of the tables reveals that in lesions resulting from $10^{-3}$ cc. inocula there is a disproportionate drop in the percentage of secondary reaction in passing from Type I to the non-fermenting members of Group X. A possible explanation for this is found in the comparison of the primary reactions. The lesions resulting from the inoculation of $10^{-1}$ cc. of Type I culture into a normal animal are comparatively large, measuring frequently 30 mm. or more lengthwise by 15–20 mm. in breadth, and often 3–4 mm. in thickness. They are deeply congested and considerably infiltrated, and are usually surrounded by an area of slight edema. The $10^{-3}$ cc. lesions are at first at least 1 mm. in thickness, and remain measurable usually for 4 to 5 days. As a rule it may be said that the strains of Group X produce smaller primary lesions, and that the smallest of all

are given by those which fail to ferment inulin. Examples of the latter have been encountered, $10^{-1}$ cc. of which failed to cause a lesion larger than that produced by $10^{-3}$ cc. of a Type I strain. These differences are too great to be explainable upon the basis of growth inequalities, and would appear rather to indicate that the Type I organisms possess primarily a greater toxicity for tissue. Either upon this or some similar factor, or upon the relative size of the primary lesion, or upon both combined, the appearance of the secondary reaction would seem to depend.

The above statements must be understood to refer to general trends. Specifically, a certain amount of variability has been found in the capacity of the strains of any one group to yield the secondary reaction. It affects the organisms of Type I, however, less than those of Group X. The reasons for this variability are not known. It seems to be independent of the source of the culture, its luxuriance of growth, or possibly its capacity for toxin production.

The recognition of variability in two factors, *viz.*, in the allergizing capacity of different strains of streptococci and in the different reacting capacity of rabbits is most important, not only from the viewpoint of simple experimentation, but from that of infection and immunity. As is indicated elsewhere (7) the degree of reactivity to reinfection is conditioned to a considerable extent by the type of response the animal exhibits following the primary infection; hence if a poorly reacting animal is infected with a strongly allergizing strain its subsequent reactivity may be similar to that of a strongly reacting animal primarily infected with a weakly allergizing strain. When, on the other hand, both parasite and host possess in a high degree the substances or qualities favorable for production of hypersensitiveness, subsequent reinfections will stimulate the most marked response; and finally if neither possesses these qualities the minimum of effect from subsequent reinfections can be anticipated. It is, of course, well recognized that these two factors may, and probably do, play an important rôle in the infectious diseases; but such variability in the two elements, host and parasite, are rarely so readily demonstrable as in these experiments.

This point is further emphasized by comparison of certain indifferent and green streptococci.

Positive ophthalmic tests (3) are readily obtainable in animals properly sensitized by the intradermal route with Type I strains. They have frequently been observed following a single series of injections, the total dosage of which has not exceeded 0.333 cc. of Type I culture. The production of a comparable degree of hyperergy with *Streptococcus viridans* requires a definitely greater initial dosage of culture, and, unless this dosage is rather large, a somewhat more protracted period of sensitization. The indifferent streptococci of Type I are therefore rather more efficient allergizing organisms than are those of the *viridans* group, in that smaller intradermal dosages will give rise in a shorter time to a demonstrable condition of hyperergy.

A positive lethal test (3), while not obtainable with the same constancy as the ophthalmic reaction, is occasionally encountered even with animals sensitized by a single series of intradermal injections of Type I strains, the total dosage of which does not exceed 0.333 cc. At autopsy the findings are similar to those which have been described in previous reports (3) dealing with *Streptococcus viridans*; namely, enlarged and congested lymph nodes and thymus with hemorrhages in these organs, in the bone marrow and elsewhere. In addition, striking gross edema and congestion of the lungs are encountered. These findings differ radically from those which follow the intravenous injection of the organisms into normal animals; in the latter case, death occurs only after a period of several weeks, and the autopsy reveals the lesions of vegetative endocarditis which have been described by other observers (4).

## SUMMARY AND CONCLUSIONS.

The indifferent streptococci are remarkably efficient allergizing agents when inoculated intradermally into rabbits.

This is revealed by the high percentage of secondary reactions which occur in the lesions resulting from the inoculation of small doses of these organisms, and by the relative frequency with which positive ophthalmic reactions are obtained following sensitization with relatively small doses.

This allergizing capacity is most marked in the organisms of Type I and least marked in the non-inulin-fermenting strains of Group X.

The different resultants emerging from variations in allergizing capacity of streptococci and reactivity of host are clearly demonstrated in this series of experiments.

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# TRANSMISSION OF FOWL-POX BY MOSQUITOES.

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Plates 38 to 41.

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Fowl-pox, a common disease of the barnyard, annually recurs in epidemic form throughout numerous countries. The virus that produces the disease is an infectious agent (1) capable of passing through bacteria-tight filters (2), and, though not identical with other pox-producing viruses, probaby is closely related to some of them. As a rule fowl-pox is not extremely fatal, yet the depression in the egg-laying activity of infected fowls leads to a great economic loss.

From the records of the epidemics of fowl-pox studied by Bollinger (1), Sanfelice (3), and others, it appears that the disease occurs for the most part during the spring and fall months. Summer epidemics, however, have been observed. In America, according to Beaudette (4), the disease is most prevalent during April and October.

Although the fact that fowl-pox is transmissible by direct inoculation (1) has long been recognized, the mode of its rapid spread under natural conditions has remained somewhat of a mystery. Contact infection undoubtedly can occur at times. Bollinger (1) reported that he obtained an infection in a healthy chicken by placing it in a yard with a sick fowl. Burnet (5), however, was able to obtain results similar to Bollinger's only when the majority of the feathers had been plucked from the healthy fowl before it was placed in contact with the diseased one. He was also able to infect a pigeon by feeding it virus, but, in order to assure a positive result, he mixed ground glass with the meal of wheat and virus. In this instance the lesions occurred in the mouth and esophagus and not on the comb, the usual place for the disease to manifest itself. Notwithstanding the results above described, the experience of most workers (Burnet (5)) has been that under laboratory conditions healthy fowls housed in the same cage with diseased birds do not contract fowl-pox. Indeed, Goodpasture (6) states that no method of artificial infection seems adequate to explain the rapid and thorough infestations which occur under natural conditions.

Some workers have attempted to incriminate (7) various insects as vectors of fowl-pox. Mégnin (8) suggests that flies and other insects may play a rôle in the dissemination of the disease, but offers no convincing experimental evidence

in support of his hypothesis. Farmers (4, 9) in various localities are of the opinion that mosquitoes are instrumental in the spread of the malady, but no records of experiments substantiating the idea have been found. Schuberg and Kuhn (10), however, report that in each of 3 tests they succeeded in transferring fowl-pox from diseased to normal chickens by means of interrupted feedings of *Stomoxys calcitrans*,—the flies starting their meal on an infected bird and completing it on a healthy one. These experiments were not extended, and it appears that they have aroused little, if any, interest.

The records of failures to transmit fowl-pox under the conditions indicated above, the occurrence of epidemics in the spring, summer, and fall, the fact that the lesions appear on exposed parts of the body (comb and wattles), and the experiments reported by Schuberg and Kuhn (10) indicated to us that biting insects may play an important rôle in the transmission of the disease. The experiments described in the present paper were undertaken to ascertain whether mosquitoes are of any significance in the spread of fowl-pox.

## EXPERIMENTAL.

### *Methods and Materials.*

*Virus.*—The strain of fowl-pox virus employed was originally obtained from Dr. Andervont and has been in this laboratory for approximately 2 years. Before starting our experiments we tested the activity of the virus by means of 2 serial passages in chickens, and also proved its purity by suitable tests in rabbits.

*Chickens.*—With a few exceptions the chickens employed in the experiments were White Leghorns. In working with fowl-pox one must remember that it is a natural disease of chickens and that immune or partially immune fowls are likely to be encountered. Consequently experiments should be repeated frequently or should be run in duplicate.

*Mosquitoes.*—*Culex pipiens* and *Aëdes ægypti* were used. The former were either caught in houses or bred in the laboratory from larvæ; the latter were hatched in the laboratory from dried eggs supplied by Dr. Boyd of the Rockefeller Foundation.

*Cages.*—The mosquito cages consisted of stiff wire frames made in the form of a cube and covered with ordinary cheese cloth. From one side of the cube the cloth extended in the form of a sleeve which served as the only opening to the cage and through which the mosquitoes or larvæ were manipulated.

*Technique of Feeding.*—Individual mosquitoes were caught in wide test-tubes, 8″ x 1″. The mouth of each tube was then covered with cheese cloth, which was held in place by a rubber band. Since mosquitoes do not feed readily under these conditions, they were starved for 24 hours prior to the experimental meal. Feeding

was further facilitated by first holding the gauze-covered end of the tube containing the mosquito towards the source of light. The mosquito was attracted by the light and came to rest on the gauze. The tube was then manipulated without disturbing the mosquito so that the gauze-covered mouth of the tube was placed over the area of the comb or wattle to be fed upon. If the mosquito was hungry and its proboscis gently came in contact with the skin of the chicken, feeding promptly ensued. Sometimes a great deal of patience was required. As a rule, however, no great difficulty was encountered in getting the mosquitoes to feed on or near lesions.

The feeding once begun could be interrupted at any time by gently lifting the tube. To prevent infection arising from the apparatus, immediately after the feeding had been interrupted the gauze was removed, the lip of the tube was washed with alcohol and dried, and a clean piece of gauze was placed over the mouth of the tube. At different intervals of time following the infectious meal the mosquito was made to refeed on normal chickens. During the process of refeeding the insect often interrupted its meal either because of an unsatisfactory probe or because the operator moved the tube. If the interruption was brought about so gently that the mosquito remained on the gauze the meal was immediately resumed at a new place. The natural or imposed interruptions proved to be important because in this manner a single mosquito has been shown to be able either to infect a fowl in several places or to infect more than one fowl.

Each tube and each chicken were numbered and records were kept of the time and place of the different feedings. After the completion of each meal the tubes containing the mosquitoes were placed upright in a wire basket and covered with a moist towel. In order to determine the survival time of the virus, the infected mosquitoes were fed on normal chickens at intervals of 3 or 4 days. At various intervals of time following the infecting meal, mosquitoes were also killed, macerated, and injected into the comb or wattles of a fowl.

Eighteen experiments were conducted, 10 with *Culex pipiens* and 8 with *Aëdes ægypti*. The results are shown in the following protocols.

## *Culex pipiens.*

*Experiment 1.*—Sept. 26, *Culex* 2 fed on the infected comb of Chicken 46, drawing whitish fluid. 1 hour later the insect made several insertions of its proboscis, without feeding, on the left side of the comb of Chicken 47. Oct. 5, 4 small vesicles were observed at the points where the mosquito had attempted to refeed. Oct. 8, lesions larger (Fig. 1). Fluid from two of the vesicles was removed by means of a capillary pipette and inoculated on the scarified comb of Chicken 56. 4 days later the comb of Chicken 56 showed definite lesions which progressed and endured for many days; Figs. 4 and 5 are photographs of Chicken 56 taken a week and a month, respectively, after inoculation. Oct. 10, lesions still increasing in size (Fig. 2). Oct. 22, lesions still present (Fig. 3). Oct 25, lesions regressing.

*Experiment 2.*—Sept. 26, *Culex* 3 fed on the infected comb of Chicken 46, drawing blood. 45 minutes later the insect refed on the right side of the comb of Chicken 47. No lesions developed at the points of refeeding.

*Experiment 3.*—Sept. 26, *Culex* 4 fed on the infected wattle of Chicken 45, drawing blood. 45 minutes later the mosquito refed on the right wattle of Chicken 47. No lesions developed at the points of refeeding.

*Experiment 4.*—Sept. 28, *Culex* 5 fed near lesions on the infected comb of Chicken 46, drawing blood, and 15 minutes later refed, 1 bite, on the left wattle of Chicken 51. Oct. 5, a vesicle appeared on the wattle of Chicken 51 at the point where the mosquito had refed 7 days previously. Oct. 11, lesion larger (Fig. 6). Oct. 22, small secondary nodules were observed on the wattle (Fig. 7). The chicken was tested, Nov. 10, for immunity to fowl-pox virus.

*Experiment 5.*—Sept. 28, *Culex* 6 fed near lesions on the infected comb of Chicken 46, drawing blood. 15 minutes later the insect refed, 3 bites, on the left comb of Chicken 51. Oct. 5, 2 vesicles were noticed on the comb of Chicken 51, where the mosquito had refed. Oct. 11, lesions increasing in size (Fig. 6). Oct. 22, lesions still increasing in size (Fig. 7). Oct. 26, lesion regressing. Nov. 10, Chicken 51 was reinoculated with fowl-pox virus. Abortive lesions appeared rapidly and were gone within 9 days after the reinoculation.

*Experiment 6.*—Oct. 15, *Culex* 7 fed on the infected comb of Chicken 57, and 1 hour and 30 minutes later refed, 2 bites, on the left wattle of Chicken 59. Oct. 25, on the left wattle, at the points where the mosquito had refed, 2 vesicles were observed. The lesions progressed and were active for many days (Fig. 8). Nov. 17, definite evidence of healing.

*Experiment 7.*—Oct. 15, *Culex* 8 fed on the infected comb of Chicken 57, and 1 hour and 30 minutes later refed, 1 bite, on right side of the comb of Chicken 59. Oct. 22, a vesicle was noticed on right side of comb at the point where the insect had refed. The lesion progressed and was active for many days. Nov. 17, definite evidence of healing.

*Experiment 8.*—Oct. 18, *Culex* 9 fed on the infected comb of Chicken 57. 30 minutes later the insect finished its meal on the right wattle of Chicken 59. Oct. 25, at the points where the mosquito had bitten Chicken 59, 2 lesions, which progressed and evidenced activity for many days, were seen. Nov. 17, definite evidence of healing.

*Culex* 9 was kept alive for further refeedings.

Oct. 22, *Culex* 9 refed on the right wattle of Chicken 63. No lesions developed. This chicken was tested, Nov. 10, and found to be immune to fowl-pox.

Oct. 25, *Culex* 9 refed on the right side of comb of Chicken 66. A questionable lesion appeared, Oct. 29, and regressed in a few days. This chicken was tested, Nov. 10, and found to be immune to fowl-pox.

Oct. 30, *Culex* 9 was macerated and injected into the comb of Chicken 69. No lesion developed. This chicken was tested, Nov. 10, and found to be immune to fowl-pox.

It appears that the refeedings of *Culex* 9 on Oct. 22 and 25 took place on immune fowls. The chicken into which the macerated insect was injected also seems to have been immune.

*Experiment 9.*—Oct. 18, *Culex* 10 fed on the infected comb of Chicken 57, and 30 minutes later finished its meal on the left side of the comb of Chicken 59. Oct. 25, 2 small lesions were observed on the left side of the comb of Chicken 59. These lesions progressed and remained active for many days (Fig. 8). Nov. 17, definite evidence of healing.

*Culex* 10 remained alive and was refed on 3 occasions.

Oct. 25, *Culex* 10 refed on the right side of the comb of Chicken 63. No lesions developed. This chicken was tested, Nov. 10, and found to be immune to fowl-pox.

Oct. 31, *Culex* 10 refed on the right side of the comb of Chicken 69. No lesions developed. This chicken was tested, Nov. 10, and found to be immune to fowl-pox.

Nov. 1, *Culex* 10 refed on the anterior part of the right wattle of Chicken 70. After being refed the mosquito was macerated and injected into the right side of the comb of the same fowl. No lesion developed at the point of injection, but on Nov. 5, 2 definite lesions were observed at the points where the insect had refed. These lesions progressed and were photographed, Nov. 7 (Fig. 10). On Nov. 9, 1 of the lesions was excised and fixed in Zenker's fluid. Stained sections showed a typical fowl-pox lesion with Bollinger bodies in the epithelial cells (Fig. 14). Material from the other lesion was inoculated on the scarified comb of Chicken 73. Typical fowl-pox lesions developed.

The lesion on Chicken 70 that was not excised and from which material was removed for transfer began to regress on Nov. 12 and by Nov. 19 was completely healed. The lesion appeared quickly and healed rapidly. It is possible that Chicken 70 was partially immune.

From the experiments recorded above it is evident that *Culex* mosquitoes are able to transmit fowl-pox from infected to susceptible normal chickens. In 7 of 9 tests, infection occurred in the susceptible fowls which had been bitten by mosquitoes that had fed on infected birds 15 minutes to 2 hours previously. In 1 of 2 experiments, *Culex* 10, in which the mosquitoes remained alive for a number of days, a typical infection was produced in a fowl 14 days after the insect had taken its infective meal. The negative results obtained with the other mosquito, *Culex* 9, were probably due to the fact that the chickens on which it refed were immune to fowl-pox.

### *Aëdes ægypti.*

Experiments similar to those with *Culex* mosquitoes were conducted with *Aëdes*. The results are recorded below.

*Experiment 10.*—Oct. 22, *Aëdes* 1 fed on the infected comb of Chicken 57. 2 hours later the insect completed its meal on the left wattle of Chicken 63. No lesions developed. This chicken was tested, Nov. 10, and found to be immune to fowl-pox.

Oct. 24, *Aëdes* 1 refed on the left wattle of Chicken 66. No lesion developed. This chicken was tested, Nov. 10, and found to be partially immune to fowl-pox.

Oct. 30, *Aëdes* 1 refed on the left wattle of Chicken 68. On Nov. 5, a lesion appeared where the insect had fed, and subsequently developed into a typical fowl-pox or contagious epithelioma wart.

After *Aëdes* 1 had fed, Oct. 30, on Chicken 68, it was macerated and injected into the left side of the comb of Chicken 68. On Nov. 16, a typical fowl-pox lesion was observed at the point where the injection was made.

*Experiment 11.*—Oct. 22, *Aëdes* 2 fed on the infected comb of Chicken 57, and 30 minutes later completed its meal on the left side of the comb of Chicken 63. No lesions developed. This chicken was tested, Nov. 10, and found to be immune to fowl-pox.

*Experiment 12.*—Oct. 24, *Aëdes* 3 fed on the infected comb of Chicken 57. The insect finished its meal 1 hour later on the left side of the comb of Chicken 66. No lesions developed. This chicken was tested, Nov. 10, and found to be partially immune to fowl-pox.

Oct.26, *Aëdes* 3 refed on the left side of comb of Chicken 67. No lesions developed. This chicken was tested, Nov. 10, and found to be immune to fowl-pox.

Oct. 29, *Aëdes* 3 refed on the posterior portion of the right side of the comb of Chicken 68. On Nov. 7, 3 vesicles appeared where the mosquito had fed and later developed in typical fowl-pox warts. Nov. 12, photographed (Fig. 9).

Oct. 31, *Aëdes* 3 was macerated and injected into the right wattle of Chicken 72. No virus of fowl-pox could be detected in the wattle excised 9 days later.

*Experiment 13.*—Oct. 24, *Aëdes* 4 fed on the infected comb of Chicken 57, and 1 hour later completed its meal on the left side of the comb of Chicken 66. No lesions developed. This chicken was tested, Nov. 10, and found to be partially immune to fowl-pox.

Oct. 26, *Aëdes* 4 refed on the right side of the comb of Chicken 67. No lesions developed and the chicken was later found to be immune.

*Experiment 14.*—Oct. 25, *Aëdes* 5 fed on the infected wattle of Chicken 51, and a few minutes later finished its meal on the right side of the comb of Chicken 66. No lesions developed and the chicken subsequently was found to be immune to fowl-pox.

*Experiment 15.*—Oct. 26, *Aëdes* 6 began its meal on the infected wattle of Chicken 61 and finished it, 2 bites, on the right wattle of Chicken 67. A doubtful lesion appeared, Nov. 3, and disappeared within a few days. Chicken 67 was tested, Nov. 10, and found to be immune to fowl-pox.

Oct. 28, *Aëdes* 6 refed in the center of the right side of the comb of Chicken 68.

No lesions appeared. Chicken 68 was definitely susceptible (see Experiments 10 and 17).

Oct. 31, *Aëdes* 6 refed on the left wattle of Chicken 69. No lesions developed and the fowl was later found to be immune.

Nov. 3, *Aëdes* 6, with *Aëdes* 8, was macerated and injected into the right side of the comb of Chicken 72. No lesions developed.

*Experiment 16.*—Oct. 26, *Aëdes* 7 fed on the infected wattle of Chicken 61, and a few minutes later finished its meal on the left wattle of Chicken 67. On Nov. 1, a small lesion appeared which persisted for only 3 days. Chicken 67 was tested, Nov. 10, and found to be immune to fowl-pox.

Oct. 29, *Aëdes* 7 refed on upper part of the right wattle of Chicken 68. No lesion developed. The chicken was definitely susceptible (see Experiments 10 and 17).

Oct. 31, *Aëdes* 7 refed on the central portion of the right side of the comb of Chicken 69. No lesion developed. The fowl was subsequently shown to be immune to fowl-pox.

Nov. 3, *Aëdes* 7 was macerated and injected into the left wattle of Chicken 72. No fowl-pox virus was demonstrated in the wattle excised 9 days later.

*Experiment 17.*—Oct. 26, *Aëdes* 8 fed on the infected wattle of Chicken 61. A few minutes after the meal was interrupted it was completed on the upper part of the left wattle of Chicken 67. No lesion appeared. Chicken 67 was shown subsequently to be immune.

Oct. 29, *Aëdes* 8 refed on the lower part of the right wattle of Chicken 68. On Nov. 3, 2 small nodules were observed at the points where the insect had refed. Nov. 12, lesions photographed (Fig. 9). These nodules developed into typical fowl-pox lesions.

Nov. 1, *Aëdes* 8 refed on the posterior part of the right wattle of Chicken 70. No lesion developed. This chicken was certainly partially susceptible at the time it was bitten (see Experiment 9).

Nov. 3, *Aëdes* 8, with *Aëdes* 6, was macerated and injected into the right side of the comb of Chicken 72. No lesion developed.

The results of the experiments with *Aëdes* mosquitoes were not as uniformly positive as were those with the *Culex*. Eight *Aëdes* mosquitoes were fed on infected chickens and later, at different intervals of time following the infective meal, were refed on healthy fowls. Of the 8 refeedings that were made within 2 hours of the infective meal only 2, *Aëdes* 6 and 7, resulted in lesions at the points where the insects bit. These lesions were small and disappeared within a few days. Refeedings of *Aëdes* 1, 3, and 8, which took place 9, 5, and 3 days respectively after the primary feeding, gave rise to typical fowl-pox lesions. The many negative results obtained in these experiments

were probably due to the fact that 4 of the 6 chickens, 63, 66, 67 and 69, employed were subsequently found to be either partially or completely immune to fowl-pox. Sufficient positive results were obtained, however, to show that *Aëdes* mosquitoes are capable of inoculating healthy susceptible chickens with the virus of fowl-pox many days after they have fed on infected combs of diseased birds.

*Transmission of Fowl-Pox by Mosquitoes under Natural Conditions.*

The results of the work reported above show that *Culex* and *Aëdes* mosquitoes are capable of transmitting fowl-pox. The tests, however, were made under experimental, not natural, conditions. It seemed advisable, therefore, to conduct at least 1 experiment in which the conditions approximated those occurring in nature.

*Experiment 18.*—Oct. 15, 2 healthy chickens (Nos. 61 and 62) and 1 (No. 57) with fresh fowl-pox lesions on both sides of the comb and on both wattles were placed in a mosquito-proof cage (Fig. 12). Five recently hatched *Culex* mosquitoes were also introduced into the cage. Oct. 22, 7 days after the experiment was started, 2 small lesions were observed on the lower part of the left wattle of Chicken 61. Oct. 26, lesions on Chicken 61 were still increasing in size (Fig. 11). One of the lesions was removed and fixed in Zenker's fluid. Stained sections (Fig. 13) showed a typical early fowl-pox lesion with many Bollinger bodies in the injured epithelial cells. The other lesion which was not excised went through the evolution usually observed in fowl-pox.

Chicken 62 never developed any lesions. This may have been due to the fact that it was immune to fowl-pox before the experiment was begun. In any event, it was tested, Nov. 10, and found to be immune at that time. It is unfortunate that tests for immunity cannot be made before the fowls are used for experimental purposes. Chicken 61, that showed the 2 typical lesions, later developed an immunity, which was probably acquired through the infection contracted during the experiment.

The control for the experiment was conducted in a manner similar to that employed in the test, with the exception that mosquitoes were excluded from the cage. 2 healthy chickens (Nos. 77 and 79) were placed in a cage with a fowl (No. 73) that had fresh fowl-pox lesions on the comb. The healthy chickens frequently pecked at the warty growths on the comb of the infected fowl. In spite of this close contact with the infectious agent, the healthy birds developed no lesions during the 12-day period of observation. They were later shown to be susceptible to fowl-pox.

The results of Experiment 18 clearly indicate that mosquitoes, under natural conditions, can transmit fowl-pox from diseased to healthy susceptible chickens.

*Nature of the Lesions Produced by Insect Inoculation.*

To present a study of the pathology of fowl-pox is not the purpose of this paper. It seems desirable, however, to describe the lesions produced by insect inoculation.

At the points on the comb or wattles of susceptible chickens where infected mosquitoes had fed or attempted to feed, minute nodules usually appeared within 5 to 9 days. Such lesions rapidly increased in size and assumed a grayish, glistening, translucent appearance. Frequently within 2 or 3 days the nodules were partially transformed into vesicles from which, by means of a capillary pipette, small amounts of whitish fluid rich in virus could be obtained (Figs. 1, 10, 11). If undisturbed the lesions continued to increase in size and gradually became large, yellowish, warty growths (Figs. 3 and 7). Finally regression and healing supervened and the wart-like scabs fell off, leaving superficial white scars. In non-immune fowls with 2 to 8 lesions, the duration of the disease was 3 to 6 weeks. In what appeared to be partially immune chickens, however, the incubation period was short, only 4 days at times, and the evolution of the disease was rapid, frequently requiring less than 2 weeks (see Experiment 9).

In certain instances, the stained sections of young lesions presented a marked hyperplasia of the epithelial cells with little, if any, reaction in the corium (Fig. 13*a*). In the case of 1 fowl, however, a lesion, approximately of the same age as the ones above described showed, in addition to the hyperplasia, vesicles in the epithelial layer and a marked cellular reaction in the corium (Fig. 14*a*). An explanation of this difference in the amount of reaction in the corium is not possible at present. Possibly this difference can be accounted for upon the ground that the depth of the insertion of the insects' proboscis varied considerably with each feeding and attempted feeding or that the reactivity of the fowls, without respect to specific resistance or immunity, was not the same in every case. Our impression, however, is that the marked early reaction in the corium occurred in a partially immune fowl (Experiment 9). This idea is at least in agreement with some observations recently reported by Andrewes (11) in regard to the histology of Virus III lesions in partially immune rabbits. From our studies it is clear that in some early fowl-pox lesions produced by the bites of infected mosquitoes a marked involvement of the epithelial cells may occur before any definite evidence of a reaction is discernible in the corium.

## DISCUSSION.

To incriminate an insect as an important vector of disease, it is essential to establish the fact that it bites readily and comes in close contact with the susceptible hosts. The intimate relation between fowls and mosquitoes has long been recognized. Furthermore, *Culex* mosquitoes feed readily on birds and are known to transmit bird malaria.

Under the conditions of the experiments reported in the present paper it is evident that *Culex* and *Aëdes* mosquitoes are capable of transmitting fowl-pox from diseased to healthy susceptible chickens. Moreover, the indications are that these insects may play an important rôle in the rapid and thorough infestations of flocks under natural conditions. The question, however, as to whether this mode of transmission is the most significant one remains to be answered by experiments conducted in the field. Certainly there is no reason why other biting insects may not also be of importance (10).

While in most epidemics of fowl-pox the lesions occur on the chickens' comb and wattles, in occasional outbreaks the majority of the manifestations of the disease is found in the mouth and throat. Inasmuch as the mucous membranes are inaccessible to mosquitoes and since the eating of virus alone usually does not result in infection, the spread of this type of the disease is hard to relate either to mosquitoes or to the simple ingestion of virus. It is possible that another infection, *e.g.*, bacterial, may injure the mucous membranes, thus making them susceptible to fowl-pox. In this manner a combination of infectious agents may account for the unusual and frequently fatal form of the disease.

It has been shown that mosquitoes are capable of transmitting fowl-pox at various times during the first 14 days following the infective meal. Tests at intervals longer than 2 weeks were not made. Therefore, the total duration of infectiousness of the insects remains to be determined. In view of the fact that the virus is active in minute quantities and is highly resistant to drying, the results of our experiments can be explained entirely upon the grounds that the mosquitoes mechanically transmit the disease without the occurrence of any multiplication of the virus in the insects. Before definite conclusions

can be reached, however, this phase of the problem will require further investigation.

The fact that insects may play a definite part in the rapid spread of certain diseases by mechanically transferring the virus from diseased to healthy individuals has been recognized by other investigators (13–16). This applies particularly to the virus diseases of plants (17). Nevertheless, from the results of our experiments, it appears that the importance of this mode of dissemination of certain virus diseases (12) of animals has either been underestimated or not sufficiently investigated.

## CONCLUSIONS.

*Culex* and *Aëdes* mosquitoes are capable of transmitting fowl-pox from diseased to healthy susceptible chickens.

The mosquitoes remain infectious for at least 14 days following a meal on diseased fowls.

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## EXPLANATION OF PLATES.

### PLATE 38.

FIGS. 1 to 3. Photographs of Chicken 47, showing the development of 4 fowl-pox lesions caused by the bites of an infected *Culex* mosquito.

FIGS. 4 and 5. Photographs of Chicken 56 that was inoculated with infectious material taken from the lesions on Chicken 47.

### PLATE 39.

FIGS. 6 and 7. Photographs of Chicken 51, showing the development of fowl-pox lesions induced by the bites of infected *Culex* mosquitoes. *a* is primary lesion; *b* are secondary nodules.

FIG. 8. Chicken 59 with lesions produced by the bites of infected *Culex* mosquitoes.

FIG. 9. Chicken 68 with lesions induced by the bites of infected *Aëdes* mosquitoes.

FIG. 10. Chicken 70 with 2 young fowl-pox lesions on the wattle induced by the bite of *Culex* 10, 14 days after its infectious meal. Lesion *a* was removed for histological study (see Fig. 14).

FIG. 11. Chicken 61 with 2 young fowl-pox lesions on the wattle. The bird contracted the disease in a cage in which healthy and infected fowls were placed with *Culex* mosquitoes. Lesion *a* was removed for histological study (see Fig. 13).

### PLATE 40.

FIG. 12. The cage in which the transmission of fowl-pox by mosquitoes under natural conditions was tested.

### PLATE 41.

FIG. 13. *a* represents a section of a young fowl-pox lesion taken from Chicken 61. Note the hyperplasia of the epithelial cells and the absence of reaction in the corium. × 50. *b* represents a normal epithelial cell. × 1200. *c*, *d*, *e* represent epithelial cells with Bollinger bodies in their cytoplasm. × 1200.

FIG. 14. *a* represents a section of a young fowl-pox lesion removed from Chicken 70. Note the hyperplasia of epithelial cells, the vesicles in the epidermis, and the marked reaction in the corium. × 50. *b*, *c*, *d*, *e* represent epithelial cells with Bollinger bodies in their cytoplasm. × 1200.

(Kligler, Muckenfuss, and Rivers: Fowl-pox.)

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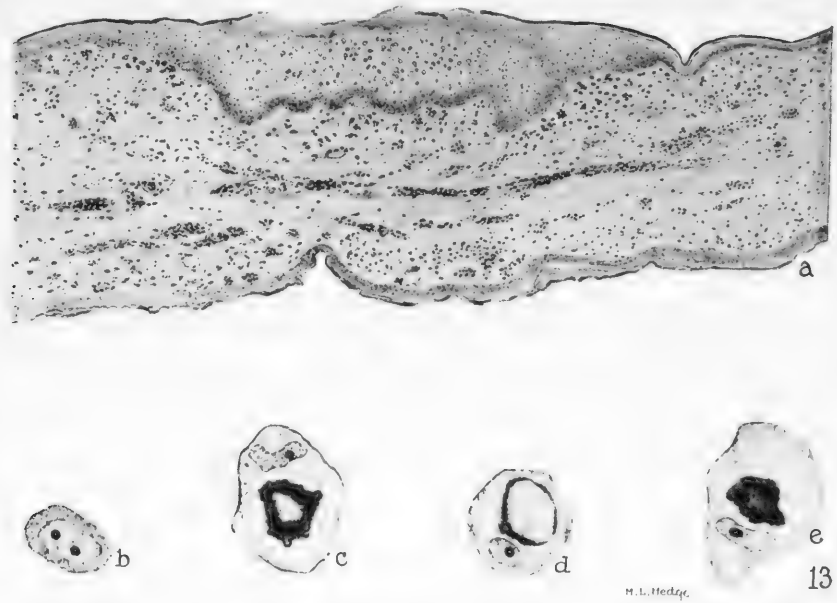


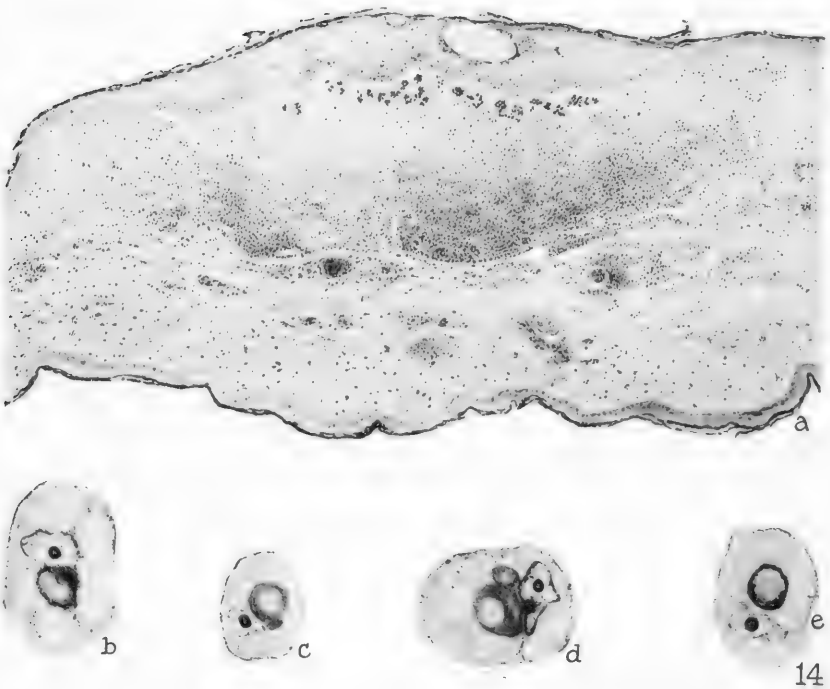


(Kligler, Muckenfuss, and Rivers: Fowl-pox.)

# HERPES ENCEPHALITIS IN CEBUS MONKEYS.

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PLATES 42 AND 43.

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For a considerable number of years we have been attempting to produce herpetic encephalitis in monkeys, at the same time that we were trying to transfer encephalitis with human material to these animals and rabbits. In spite of the occasional reports in the literature that *Macacus rhesus* and other species of *Macacus* were susceptible to herpes virus, we were entirely unsuccessful in producing any sort of a disease in the *Macacus* species, even with the most potent herpetic strain in our possession. We administered the virus intracerebrally, intraperitoneally, and implanted it in large amounts, together with agar, under the skin. Never did we obtain any reaction in these monkeys. During the past winter, having in our possession a ringtail monkey of the South American *Cebus olivaceus* species, we inoculated this animal with herpes intracerebrally, and found that a disease not unlike that occurring in rabbits killed this animal in the course of 9 days. Since that time we have attempted to produce encephalitis in *Cebus olivaceus* monkeys with the spinal fluid of several cases diagnosed as probable encephalitis, but apart from a curious edema of the face and temporary unwillingness to eat, in one animal, coming on about a week to 3 weeks after inoculation and from which complete recovery occurred, we obtained no results whatever.

Although a *Cebus* monkey inoculated with the spinal fluid from a case at the Boston Psychopathic Hospital did not develop any symptoms which could be regarded as simulating a true encephalitis, the subsequent inoculations of other monkeys carried out with the brain of this one developed into a series of observations which we believe sufficiently interesting and important to record in detail.

Fresh brain from the original spinal fluid monkey, which we shall call *Cebus* I, was inoculated intracerebrally[1] on May 21 into *Cebus* II and into two rabbits. The rabbits remained entirely well, not even developing a temperature. *Cebus* II likewise showed no signs of illness, except that 7 days after inoculation it developed a swollen edematous area on the right side of the face during a period of about a week. The edema then subsided and the monkey returned to normal.

On July 9, material from the first monkey, *Cebus* I, which had now been in glycerol for 21 days, was inoculated into a third monkey, *Cebus* III, and into two rabbits. These two rabbits developed—4 days after inoculation—temperatures rising to 105.1° in one case and 105.8° in the other, but recovered. Transfers to other rabbits from these animals developed no virus effects, and the monkey in this series developed no symptoms whatever.

On August 4, another monkey, *Cebus* IV, was inoculated, again with material from *Cebus* I, now 45 days in glycerol in the ice chest, and this animal also developed a facial edema with a few days of apparent illness evidenced by lassitude and loss of appetite. This monkey completely recovered within a week, and thereafter remained well.

At first we thought that the facial edema which occurred in three separate animals might have some special significance, but concluded that it probably was due to some injury not anatomically traceable, consequent upon the intracerebral inoculations. *Cebus* III, which was killed and examined, had developed upon the edematous area a small staphylococcus abcess. We therefore paid no particular attention to the edema, which we could not then and cannot now explain satisfactorily, and preserved these monkeys for subsequent inoculation with herpes virus, in order to find out whether they had developed any immunity.

The above preliminary history of these monkeys is given for the purpose of exactitude, although we are inclined to believe at the present time that the previous inoculations had little to do with the subsequent histories of the animals. This will appear below.

On October 2, *Cebus* IV, above described, now entirely well, was intracerebrally inoculated with brain material taken from Rabbit 42 and glycerolated for 1 week. This rabbit had died typically after inoculation with an herpetic filtrate. At the same time, a normal *Cebus*, *Cebus* V, was inoculated as a control. The difference between these two monkeys was a striking one, both clinically and pathologically.

*Cebus* V died as other normal monkeys of this species have died after inoculation with herpes, after an acute illness of 9 days. *Cebus* IV, however, did not show any signs of illness until the 10th day after inoculation. On October 12 it developed

[1] Ether anesthesia was used in all operative experiments.

an unwillingness to eat, tremors and lassitude. This on subsequent days developed into a state of somnolence which by October 15 and 16 was so marked that the monkey squatted in the corner of the cage, rarely changing its position, swaying to and fro with its eyes closed and obviously asleep. The only movements were occasional twitchings of the arms and legs. This condition continued day after day. When aroused and offered food, the monkey would eat and occasionally would make uncoordinated and purposeless waving movements with the hands, alternating with tremors. Immediately upon being set back into the cage, it relapsed into deep somnolence from which it rarely roused itself spontaneously. When the limbs were extended, they went into a temporary spastic condition. The

TEXT-FIG. 1. Habitual position held by *Cebus* IV while somnolent during a period beginning 12 days after inoculation and lasting until killed, 24 days after inoculation.[2]

monkey was examined during this period by Dr. Stanley Cobb and in its general clinical condition displayed a typical encephalitis of an acute type. On the 24th day after inoculation, the animal seemed to be a little more lively, and in order that we might obtain material for the study of the pathological condition as it then existed, the monkey was etherized and the nervous system removed.

Inoculations were made from the brain of *Cebus* V, which died acutely, into another monkey, *Cebus* VI, and into two rabbits—all of

[2] All the photographs were taken by Dr. S. Burt Wolbach, to whom I wish to express my appreciation and thanks.

which died acutely and with typical signs of herpes, proving that the condition of which the control monkey had died was one of herpes.

Material from *Cebus* IV, the one with prolonged somnolence, was inoculated into another monkey, *Cebus* VIII, on November 2, and this monkey has shown nothing to date. We neglected at this time to inoculate rabbits from this monkey, but when—by November 15—the virus of *Cebus* IV had failed to kill a monkey of the same species, while the virus of its acutely dead control *Cebus* V, had killed promptly, we inoculated two rabbits with the material of *Cebus* IV, since it seemed that during the chronic condition in this monkey the virus had become either attenuated or destroyed. The material by this time had been in glycerol since October 25, and of course it was an experimental error not to have inoculated rabbits immediately, with the fresh material. This rabbit died of typical, acute herpes. The problem involved will be discussed below. Text-fig. 1 is a photograph of *Cebus* IV, which remained somnolent, in the position which it held practically without change while asleep for 12 days.

The most interesting part of our experiments—indeed, the findings which encouraged us to publish them—are the pathological examinations of the brains. Although all three monkeys of these experiments had the same virus, *Cebus* IV was killed after a prolonged somnolence, whereas V and VI died acutely. The differences in the length of time for which these monkeys survived seem at the same time to have involved important differences of the pathological picture.

The following pathological report is one which was very kindly prepared for us by our colleague, Dr. Wolbach, whose assistance we solicited in order to be sure that there would be no mistake in the pathological interpretation.

*Cebus IV.*—(This is the monkey which showed no symptoms whatever for 12 days, on the 12th day developed the picture of encephalitis described above and was killed 12 days after the onset—that is, 24 days after inoculation.)

Eight sections of the spinal cord representing different levels are negative. Three sections of the cerebellum show no lesions. Two sections through the basal ganglion, presumably the caudate nucleus and underlying internal capsule, and two sections through the pons and medulla show very numerous and fairly striking perivascular infiltrations, always about blood vessels of medium size. The cells consist chiefly of lymphoid and plasma cells, but there are many cells that have the characteristics of mononuclear phagocytes, rarely, however, containing

inclusions. There are also cells that seem to be of connective tissue origin. This is borne out by the fact that there is undoubtedly an occasional minute cicatrix that has formed in the adventitia, rarely an eosinophil cell. In the immediately adjacent zone of nerve tissue there has been an increase of neuroglia and in the pons and basal ganglion remote from blood vessels of any size are small collections of neuroglia, resembling very much the late typhus lesion and in some instances the glia stars of Spielmeyer. These perivascular infiltrations are most abundant and most striking beneath the ependyma of the lateral ventricles in the basal ganglion and beneath the ependyma of the fourth ventricle. The minute glia cicatrices are found chiefly in the nuclei of the pons and in the basal ganglia on both sides of the internal capsule, and therefore presumably both in caudate and lenticular nuclei. The cerebral cortex is represented by five sections, all of which show lesions. In one section there is very striking diminution of ganglion cells, and it is possible to determine the former location of ganglion cells by clusters of neuroglia. The more acute lesions are represented by clusters of cells, the satellite cells, but throughout there is a very pronounced increase of astrocytes. In this region the perivascular infiltration is very striking. The vessels are surrounded by broad zones of cells composed of lymphoid, plasma cells and an occasional macrophage, a rare eosinophil and fibroblasts arising evidently from the adventitia of the vessel. The pia and arachnoid over this region are heavily infiltrated and here it is possible to see that there has been a very considerable increase of connective tissue, but the picture is again dominated by lymphoid, plasma cells and macrophages. There are a *few* degenerative ganglion cells in this region with greatly swollen nuclei filled with a finely granular, deeply eosin-staining material. These resemble very closely the inclusions described by Lipschütz (1) and, recently, by Goodpasture (2). The inclusions are typical of herpetic lesions in rabbits. They were described by Goodpasture in ganglion cells and in other tissues. There is a very marked increase of neuroglia cells of the astrocyte type beneath the glia, and everywhere throughout the cortex there is an increase in cell richness. Numerous cells contain large vacuoles and agree in appearances with the oligodendroglia cells which seem on the whole to be increased in numbers. To summarize, the brain of this monkey shows in basal ganglion, pons, medulla and cerebral cortex many perivascular infiltrations. In the gray matter of all these locations there are numerous minute neuroglia cicatrices. In the cortex there are more recent lesions, as evidenced by the finding of degenerated ganglion cells, and neuronophagia. The degree of perivascular infiltration and infiltration of the leptomeninges corresponds to lesions found in human cerebral trypanosomiasis. There are no vascular thromboses. Careful search fails to reveal any recognizable parasites or microorganisms of any sort.

*Cebus V.*—(This is the monkey which died of acute encephalitis 8 days after inoculation.)

There are sections from five different portions of the cortex and underlying white matter. Sections representing three different regions show a widespread

involvement of the cortex evidenced by lesions of individual ganglion cells, increase in satellite cells and an early perivascular reaction. None of these changes are conspicuous as observed through the low power. The outstanding feature to be observed with the low power is the swelling of nuclei and the presence of nuclear inclusions. Many of the ganglion cells containing these inclusions show little change. Others show very marked degenerative changes. The inclusions are those typical of the herpetic infection as described by Goodpasture, Lipschütz and many other authors. The earliest form seems to be a deeply staining, irregular, somewhat ameboid appearing inclusion. In every instance there is disappearance of the nucleolus. Later stages are represented by nuclei very markedly distended, outlined by a deeply staining, nuclear membrane, presumably containing peripherally displaced chromatin bodies. The inclusion itself in these conditions seems to consist of a mass of finely granular, neutral staining material. With the highest powers and best illumination, one gets the impression that it is composed of minute granules, tightly packed together. These degenerative ganglion cells containing these nuclear inclusions are always accompanied by several greatly enlarged satellite cells, and neuronophagia is in progress. Here and there throughout the cortex there are accumulations of cells in and about the adventitia of the blood vessels. These cells are of an indiscriminate type for the greater part and resemble macrophages or are young fibroblasts. Only occasionally can cells be identified as belonging to the lymphoid series. The appearances on the whole are those that give support to the idea that the lymphoid and plasma cells in perivascular infiltration arise from indifferent cells which take origin in the adventitia of blood vessels. There are a few foci where there is a much greater reaction, where ganglion cells are completely necrotic and surrounded by clusters of enlarged phagocytic cells. Around capillaries there are occasional polymorphonuclear leucocytes. In these areas and in less focal distributions there is a much more pronounced perivascular infiltration. Every ganglion cell is involved, —that is, containing nuclear inclusion with degeneration of the cell body. There are numerous polymorphonuclear leucocytes adjacent to the cells and migrating through the brain itself, and also in the zone of perivascular infiltration. Rarely there is an eosinophil about a blood vessel. The meninges show very little change. There is a slight infiltration with cells that appear to be of the lymphocytic series, although larger than the cells in *Cebus* IV (Fig. 3). Here again, are undifferentiated cells in the pia which could be taken either for fibroblasts or macrophages and suggest origin *in situ* of the lymphoid and plasma cell so commonly found in encephalitis of diverse causation. Without going into great detail, one can characterize this brain by saying that there is an extraordinarily widespread or diffuse involvement of the ganglion cells resulting in their degeneration, the formation of nuclear inclusion and the usual response on the part of the satellite cells. The acuteness of the process is evidenced by the appearance of polymorphonuclear leucocytes and the early reaction around blood vessels and in the meninges. If I should venture a guess, I should express the opinion that this slide indicates that the initial damage

to the brain has been in the ganglion cell of the cortex. In other words, that there has been a specific localization of the injurious agent in the cell. The other changes can be interpreted as secondary to the destruction of the ganglion cells. The suggestion by Lipschütz that these inclusions are due to the actual presence of the virus is not difficult to believe after seeing these preparations.

Figs. 1 and 2 represent the typical, perivascular infiltration in the brain of the monkey with the more slowly progressing condition, simulating human encephalitis. Fig. 4 is a high power drawing of the swollen, degenerated nuclei and nuclear inclusions found in such large numbers in the monkey which died in 8 days.

## DISCUSSION.

The observations which we have outlined reveal that *Cebus olivaceus* monkeys inoculated with the identical herpetic virus may react in two quite different ways, both clinically and pathologically, according to the acuteness with which the disease progresses. In the monkey which died acutely in 9 days, we have a clinical picture which has no definite similarity to human encephalitis, but resembles in its clinical and pathological features the condition observed in rabbits after inoculation with herpes virus. The lesions in this acute animal involve the ganglion cells and changes in the nuclei with the nuclear inclusions described by Lipschütz and by Goodpasture and Teague (3) in the brains of rabbits. The perivascular infiltrations observed in the typical encephalitic disease of man are relatively undeveloped and of a minor type.

In the more chronic form of the disease, on the other hand, in which we had a clinical picture strikingly like that of encephalitis in man, the pathology as well is one more closely resembling that of man, and although there are a few areas with nuclear inclusions and changes similar to those observed in the acute animal, the perivascular infiltrations and other changes are remarkably like those of human encephalitis.

It would appear from this that the same virus, when not acutely fatal, produces lesions quite different from those in the acute cases; the differences in picture being to some extent, therefore, dependent upon the length of time and the violence with which the agent is active in the central nervous system. In following the sequence of our experiments, the thought is obvious that perhaps *Cebus* IV, the animal which was more chronically ill and might have recovered, was partially

immunized by a preceding inoculation some months before with the brain of a monkey into which material from a case of human encephalitis had been injected. This would be significant etiologically, were it maintainable; but we are inclined to believe that the difference between this monkey and several others that have died acutely is one of variation of susceptibility in the individual animal, which becomes manifest when a virus of somewhat reduced potency is used.

Of considerable interest, also, is the fact that a *Cebus* monkey and two rabbits inoculated with the brain of the acute monkey died typically of acute herpetic encephalitis, whereas a similar monkey inoculated with the brain of the chronically diseased animal has shown no symptoms to date. This observation would indicate that there was some attenuation of the virus in the case of the more prolonged pathological reaction in this animal, a condition which has been referred to as autosterilization by Levaditi, and might explain the almost universal failure of transmission to animals with materials from human encephalitic cases. It must, however, be mentioned that subsequent inoculations of rabbits with the brain of this chronic monkey produced typical herpes encephalitis.

Our experiments have shown that, given the proper degree of balance between infectiousness and resistance in a susceptible species of monkey, herpes virus may give rise either to an acute disease resembling clinically and pathologically the acute herpetic encephalitis of rabbits, or to a more prolonged malady in which both clinical features and pathological lesions are strikingly similar to the human disease.

We have no idea that this solves the problem of the etiology of epidemic encephalitis, in the sense of an acceptance of the herpes virus as an etiological factor. It merely shows that the typical clinical and pathological pictures are as closely dependent upon acuteness or chronicity of the disease as they are upon the nature of the virus. Indeed, in the years during which we have unsuccessfully attempted to transfer human encephalitis to rabbits and monkeys, we have had occasion to obtain material from spontaneous encephalitis, from encephalitis following vaccination and from a recent case in which an apparently typical encephalitis followed treatment with phenolized rabies virus. The literature cites encephalitis as most frequently following influenza, vaccination, measles, varicella—all of them, except influenza, so

called "filtrable virus diseases," and influenza still uncertain in its etiology.

It would seem to us most rational on the basis of our own experience and the general experience of others to assume for the present that the clinical and pathological injuries grouped together as encephalitis might be due to the development of neurotropism by a number of different filtrable agents, and that the similarity in pathology is due to an analogous reaction on the part of the tissues to various members of a single group of closely related agents. The fact that Rivers and Stewart (4) have recently been able to produce encephalitis with Virus III would seem to further encourage the idea that encephalitic disease represents a clinical group in which herpes virus may be occasionally responsible, but in which the neurotropism of a considerable number of other filtrable agents is involved.

## CONCLUSIONS.

Herpes virus which ordinarily produces in *Cebus olivaceus* monkeys an acutely fatal encephalitis closely resembling in time, symptoms and pathology the acute, herpetic disease of rabbits may—in more resistant individual monkeys—lead to a more prolonged malady which, while unquestionably produced with herpes virus, simulates with considerable accuracy the human disease of acute encephalitis, in symptoms, in course and in pathological changes.

## REFERENCES.

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2. Goodpasture, E. W., *Am. J. Path.*, 1925, i, 1.
3. Goodpasture, E. W., and Teague, O., *J. Med. Research*, 1923, xliv, 121.
4. Rivers, T. M., and Stewart, F. W., *J. Exp. Med.*, 1928, xlviii, 603.

## EXPLANATION OF PLATES.

### PLATE 42.

Sections were stained both by the ordinary hematoxylin-eosin and by the Giemsa method. The latter was the method that showed cellular details most satisfactorily.

FIG. 1. Section through brain of *Cebus* IV, monkey with typical encephalitis, showing perivascular infiltration.[2]

FIG. 2. Section through the same brain as Fig. 1.

PLATE 43.

FIG. 3. Brain of a monkey with acute encephalitis. To show the condition of the nuclei and inclusion bodies.

FIG. 4. Oil immersion drawings of nuclear swellings and inclusions in the ganglion cells of the brain of *Cebus* V, the control on *Cebus* IV, but which—in contrast to *Cebus* IV—died acutely.

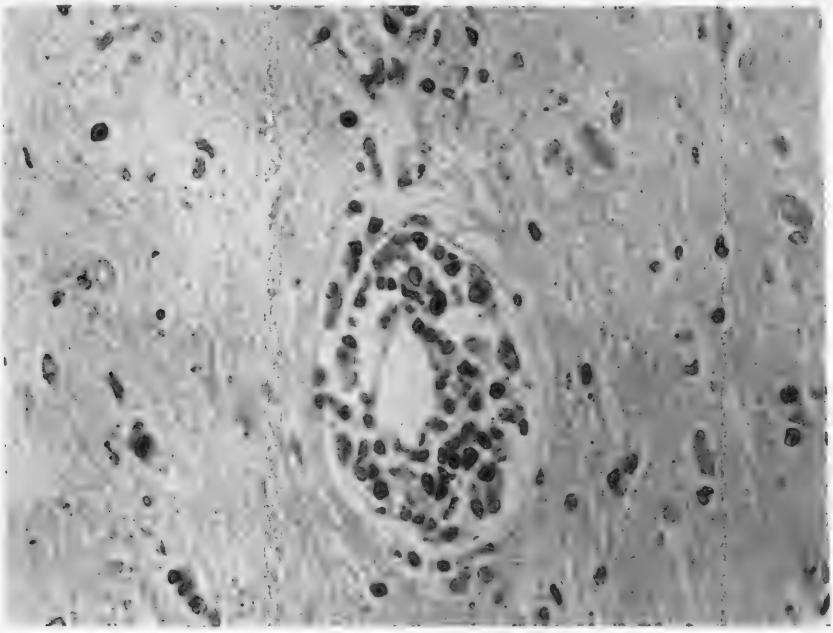

FIG. 1.

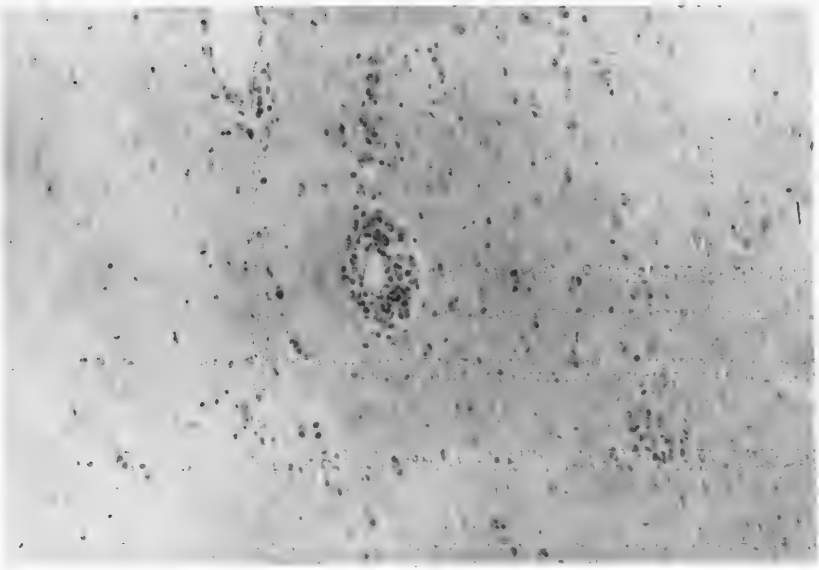

FIG. 2.

(Zinsser: Herpes encephalitis in *Cebus* monkeys.)

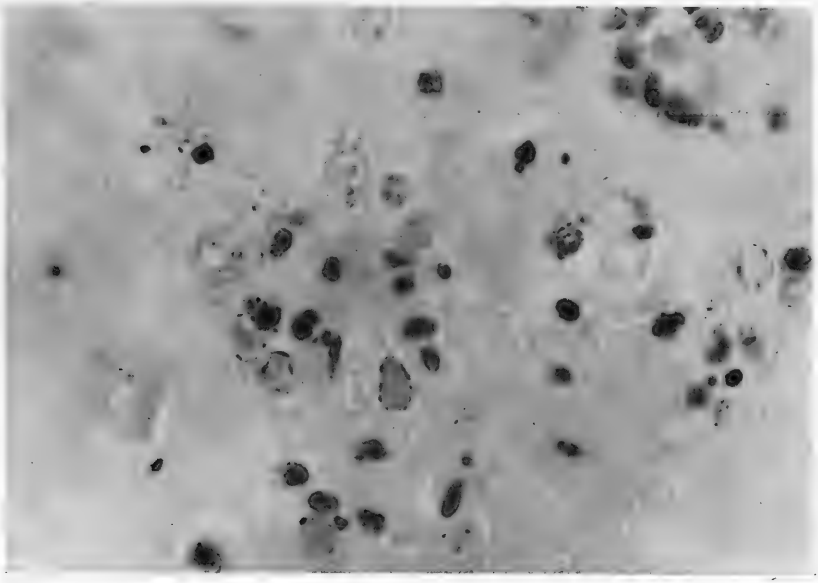

FIG. 3.

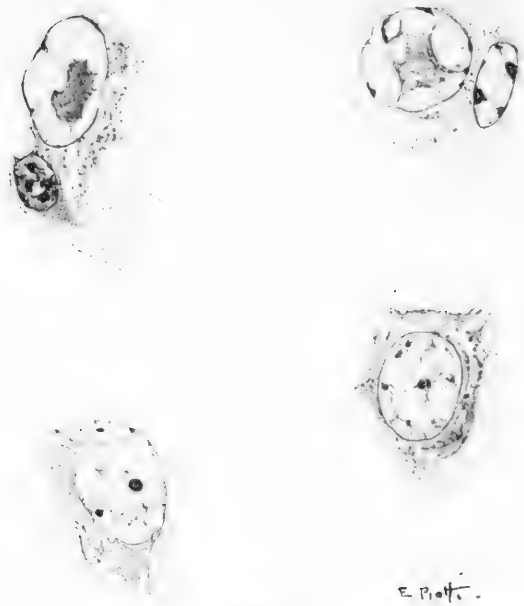

FIG. 4.

(Zinsser: Herpes encephalitis in *Cebus* monkeys.)

# A STRAIN OF BACILLUS ABORTUS FROM SWINE.

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The economic significance of abortion among swine and the evidence already presented that the particular race or races of *Bacillus abortus* causing this disease may be involved in undulant fever of man[1] justify a rather detailed study of this disease and the strains of *Bacillus abortus* associated with it.

A review of the early work with the porcine disease was published in 1926.[2] The evidence up to that date, though quite incomplete, pointed to an identity with the bovine races in terms of specific agglutinins but to differences in growth in sealed and unsealed cultures and in the lesions produced in guinea pigs. In these animals suppuration following the characteristic proliferation of monocytes into tubercle-like foci in this whole group was stressed as an important feature of the porcine races of *Bacillus abortus*. At that time no original strains were obtainable.

Late in May, 1927, our attention was called to an outbreak of swine abortion in the herd of a State institution.[3] With the effective co-operation of the State authorities we were able to obtain a few fetuses. At the same time it was learned that the animals had not been fed cow's milk and that the garbage from the institution was not heated before it was fed. The yards were infested with rats. There had been no record of abortion in this herd prior to March of this year. When it occurred the aborted fetuses and membranes appeared decomposed to the attendants. A white viscid discharge appeared around the vulva of the affected sows. There had been no fresh pigs introduced

[1] Smith, T., *J. Exp. Med.*, 1926, xliii, 207.

[2] Smith, T., *J. Exp. Med.*, 1926, xliii, 215.

[3] The writer is indebted to Dr. R. B. Little of this Department for data and material from this outbreak.

into the herd except a boar in 1926 which came from a herd free from the disease. Since the porcine disease has been subjected to but little detailed study, perhaps owing to its relatively infrequent occurrence, the notes on the several cases are briefly reproduced here.

*No. 401.*—Fetus of sow, reported to have been born dead and buried soon after. The matter was of sufficient importance at this time to warrant the recovery and study of this fetus, which had been buried overnight. Autopsy indicates that fetus had breathed. Several pieces of straw from bedding had penetrated into thorax and abdomen. Stomach empty, inflated with gas. Organs not noticeably affected. The placenta obtained in part shows irregular thin white patches of rather tough consistency, 1 to 2 cm. in diameter, which are readily peeled off the chorion. They resemble fat. Under the microscope the material composing them is amorphous and yields gas when 2 per cent HCl is passed under cover-slip. No exudates or ulcers noticed.

Owing to the condition of the fetal carcass only cultures from heart's blood and liver were made. In both blood cultures about 25 colonies appeared within 3 days. Colonies vary from ½ to 1½ mm. in diameter, and are convex, grayish, glistening. In the liver culture the same type of colony appeared. These were later identified as *B. abortus*.

*Sow 402.*—Discharged two fetuses (402a and 402b) June 2, 1927. Duration of pregnancy 7 to 8 weeks. *Fetus 402a*, 23 cm. long, from tip of snout to root of tail. Slight effusion of clear serum in serous cavities and pericardial sac. Heart pale, almost white. Liver and kidneys full of blood. Stomach contains a glass-clear thick fluid, resembling aqueous humor. In it many fecal pellets. Lungs not inflated. Only a small piece of placenta obtained. On it minute grayish deposits. A few cocci seen in films from it.

*Fetus 402b*, identical as to size and condition with 402a. About one-half of placenta obtained. The surface of the chorion is overlaid with very thin isolated grayish patches, 2 to 3 mm. in diameter. A few lumps of exudate lying on surface. Films of scrapings show masses of cells, chiefly polymorphs, many containing three or more bacilli a trifle larger than the bovine type of *B. abortus*. Some are spindle-shaped. The subchorionic tissue is slightly edematous.

Cultures from these fetuses gave the following result:

*402a.*—Cultures of spleen bit and stomach fluid sterile; of heart's blood contain a streptococcus; of lung and liver, rich growths resembling *B. coli*.

*402b.*—Cultures of stomach contents and liver remain sterile; of spleen contain a large coccus. A tube inoculated with heart's blood has a heavy growth above the condensation water; on the slope two small colonies later identified as *B. abortus*. Subcultures from these colonies develop in 24 hours without seal. The growth is moderately heavy in 48 hours.

Sections of hardened tissues from various organs of Fetuses 402a and b did not bring out any recognizable lesions. Similarly sections of the placentas were

negative. In view of the definite lesions found in the fresh material, the sections probably did not pass through any diseased areas.

*Sow 403.*—Farrowed a litter of 9 small living pigs. On the assumption that the uterus might contain *B. abortus*, a swab was introduced into the vagina to collect a whitish viscid discharge which had appeared. This was stirred in bouillon and two guinea pigs inoculated subcutaneously with the clouded suspension. When killed 29 days later both animals had gained considerably in weight. The autopsy and spleen cultures were negative in both cases.

*Sow 404.*—Farrowed a litter of 5 pigs of which 2 were born dead. These were buried together with parts of placenta. Next day, the fetuses were dug up and a uterine swab obtained from the sow.

*Fetus 404a.*—Male, 35 cm. long. Abdomen contains fluid blood. Lungs not inflated. No abnormalities noted. In making cultures bits of tissue and several drops of stomach fluid were transferred. Pure cultures of *B. abortus* were obtained from liver, heart's blood, lungs, and stomach fluid. Only a few colonies developed in spite of the large inoculum. All are convex, almost hemispherical droplets, grayish, glistening, partly translucent, measuring 1.5 to 2 mm. in diameter.

*Fetus 404b.*—Male, 32.5 cm. long. Left lung almost fully inflated; right airless. Stomach contains blood. Viscera normal in gross appearance. Both cultures containing heart's blood developed a mixed growth of several species. The liver culture remained sterile. In both tubes containing stomach contents growth appeared above the condensation water in 24 hours. On the 2nd day a heavy crop of minute colonies appeared on the slant, identified as pure cultures of *B. abortus*.

It appears from the foregoing protocols that bacilli resembling *Bacillus abortus* were isolated from the fetuses of 3 out of 4 sows. The fourth sow had a normal litter and was evidently not a carrier. Contrasting with bovine abortion, the distribution of bacilli in the fetuses was more general since cultures were readily obtainable from heart's blood and internal organs. In the bovine fetus the bacilli are in most cases limited to the digestive and pulmonary tracts and are sometimes wholly absent. The porcine fetuses examined showed no visceral lesions. The placentas obtained showed exudative processes resulting in thin excrescences. The nature of the peculiar fat-like expansions found on the placenta of No. 401 has not been elucidated.

Concerning the strains of *Bacillus abortus* obtained from the three sows or their litters, it may be stated briefly that they were identical so far as our comparative studies went. They all grew readily in

unsealed tubes and growth was visible within 2 to 3 days. The sealing had a distinctly retarding effect before or after the colonies had appeared. The rate of growth is best illustrated in the following experiment.

From a colony (401) a trace of growth is transferred on a platinum wire to 5 cc. bouillon and the latter thoroughly shaken. A loop of this suspension is transferred (*a*) to an agar slant which is sealed with sealing wax, and (*b*) to one having the lower end of the cotton plug just dipped in paraffin. To (*c*) unsealed the platinum wire transfers directly a trace from the colony. After 28 hours the bouillon suspension is distinctly clouded. (*c*) has a fairly good film. After 48 hours (*a*) and (*b*) are now covered with a dense layer of minute colonies. Those in (*b*) are 2 or 3 times the size of those in (*a*).

*Pathogenic Action on Guinea Pigs.*—Strain 401 isolated directly from a fetus was also recovered from guinea pigs as follows: The contents of a coil of the small intestine of fetus were suspended in bouillon and injected into two animals.

*Guinea Pig 4180.*—Weight 510 gm. Received subcutaneously 1 cc. suspension of fetal intestinal contents. Killed after 30 days. All subcutaneous lymph nodes swollen to about twice original dimensions. A necrotic focus in right kneefold node. A moderate number of minute grayish points showing under liver capsule. Spleen markedly congested. Dimensions twice normal. On section follicles irregularly enlarged. In two agar slants containing a bit of spleen, one sealed, growth appeared within 2 days in the unsealed tube. To hasten growth the sealed tube was pierced with a hot iron rod. 2 days later growth had begun in this tube above the bit of tissue.

*Guinea Pig 4181.*—Weight 470 gm. Chloroformed after 30 days. Lesions as in preceding animal. Cultures containing spleen bits show after 2 days about 100 characteristic colonies.

Two guinea pigs were later on inoculated with suspensions of pure cultures.

*Guinea Pig 4221.*—Received subcutaneously ½ cc. of a 1/375,000 dilution of an agar slant. The dilution is prepared by washing the entire growth into the condensation water, which is equal to ½ cc. This is further diluted to the titer indicated. Guinea pig killed after 26 days. Left kneefold and axillary nodes swollen and contain firm, yellow, 1 mm. foci. Other nodes inconspicuous. Spleen markedly congested. Dimensions twice normal. Minute grayish foci on section. Under liver capsule minute grayish foci. Cultures from spleen positive. Tubes contain several hundred typical colonies.

*Guinea Pig 4209.*—Receives the same culture but diluted only to 1/1250, into peritoneal cavity. Chloroformed in 25 days. Subcutaneous lymph nodes swollen. Spleen extremely full of blood. Dimensions 2½ to 3 times normal. Cultures as in preceding case.

*Bacillus abortus* was isolated from the uterine swab of Sow 404. The lesions produced are like those of Strain 401.

*Guinea Pig 4232.*—Receives subcutaneously ½ cc. of a turbid bouillon suspension of swab from Sow 404. Chloroformed in 21 days. At point of inoculation an abscess in the subcutis. Nearest kneefold lymph node, bean size, with still firm, necrotic foci. Spleen congested. Dimensions twice normal. Surface sprinkled over with minute grayish points. Spleen cultures contain several hundred colonies.

*Guinea Pig 4231.*—Receives 1 cc. of same suspension. Killed in 21 days. Lesions as in preceding animal with the addition of four small abscesses in omentum. Spleen and omentum cultures develop countless colonies. In cultures from both animals the growth in the sealed cultures was much retarded or else absent until seal penetrated and tubes reincubated.

Cultures of *Bacillus abortus* having identical characters in culture and in guinea pigs were thus isolated directly and through guinea pigs from 401 and 404. Material from 402 was not passed through guinea pigs.

*Agglutination Reactions.*—To determine the serological relationship between the swine strain and bovine types a culture recently isolated from a case of bovine abortion and serum from an old bovine carrier of *Bacillus abortus*, probably in the udder (No. 930), were used. The cow had carried agglutinins in her blood for a number of years. The swine sera were obtained by bleeding the sows from the tail. The absorption tests were performed as follows:

A Blake bottle containing nutrient agar was inoculated and incubated 48 hours. A suspension in normal salt solution was made which was 11 times the density of 2.4 on the Gates scale. 1 cc. of serum was mixed with 4 cc. of the suspension and centrifuged. The deposit was stirred up, incubated 2 hours, and centrifuged. The clear serum in a 1/5 dilution was tested in higher dilutions as indicated in Table I.

Reciprocal agglutination and absorption tests with a strain from swine are given in Table I.

The irregular inhibition of agglutination shown in this table is also evident in tests to be reported below. The serum of the cow is equally, but not completely, absorbed by cow and pig strain.

In Table II are recorded additional direct and cross-agglutination tests. Certain irregularities are also evident in these data. The serum from a normal pig was free from agglutinins towards swine strains. A test not tabulated showed complete absence of agglutinins

TABLE I.

*Serum of Cow 930.*

| Strains of *B. arbortus* | Dilutions | | | | | | | | | Controls (salt solution) |
|---|---|---|---|---|---|---|---|---|---|---|
| | 1/10 | 1/20 | 1/40 | 1/80 | 1/160 | 1/320 | 1/640 | 1/1280 | 1/2560 | |
| Cow 1373..... | C | ++++ | ++++ | C | C | +++ | + | – | – | – |
| Swine 402..... | +++ | +++ | ++++ | C | ++++ | ++ | – | – | – | |

*Absorption Test.*

| (Absorbed with Strain Swine 402) | Serum of Cow 930 | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Cow 1373..... | C | + | – | – | – | – | – | – | – | – |
| Swine 402..... | C | + | – | – | – | – | – | – | – | – |

TABLE II.

*Cross-Agglutination Tests (Cow and Swine Sera).*

| Serum of | Serum dilutions | | | | | | | | | Control | Strain used for agglutination |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1/10 | 1/20 | 1/40 | 1/80 | 1/160 | 1/320 | 1/640 | 1/1280 | 1/2560 | | |
| Normal pig. | – | – | – | – | – | – | – | – | – | – | Sow 401 |
| Cow 930.... | C | ++++ | +++ | ++++ | ++++ | ++++ | +++ | – | – | | " 401 |
| Swine 462... | ++++ | ++ | +++ | C | C | C | +++ | + | – | – | " 404 |
| Cow 930.... | ++++ | ++++ | ++++ | ++++ | ++++ | +++ | – | – | – | | " 404 |
| Normal pig. | – | – | – | – | – | – | – | – | – | | " 404 |
| Swine 461... | ++++ | C | C | C | C | C | ++++ | + | – | – | *B. abortus* 1373 |
| " 461... | C | C | C | C | ++++ | ++++ | +++ | – | – | – | Sow 402 |

towards the cow strain in dilutions as low as 1/10. The additional sows included in this and the following test are from the infected herd. No. 460 is a young pregnant sow, evidently not infected. No. 461 is also a young pregnant sow, showing in her serum a high agglutinin content. No. 462 is an old sow, also with a high agglutinin titer.

In Table III are given comparative tests of the swine sera with strains from swine and Cow 1373.

Here again the serum No. 460 appears free from specific agglutinins. The blood of infected Sow 402 is low in agglutinins. The rest are high. Both cow and swine strain show the irregular inhibitions with the maximum agglutination and subsidence of the bacteria around 1/160.

TABLE III.

*Agglutination of Strains from Cow 1373 and Sow 401 by Various Sera from Infected Herd.*

| Source of swine sera | Serum dilutions | | | | | | | | | Control | Strain used |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1/10 | 1/20 | 1/40 | 1/80 | 1/160 | 1/320 | 1/640 | 1/1280 | 1/2560 | | |
| B | ++ | ++ | ++++ | C | C | C | C | + | − | − | Sow 401 |
| C | ++ | ++ | C | C | C | C | ++ | − | − | | " 401 |
| 402 | ++ | ++ | ++ | ++ | − | − | − | − | | | " 401 |
| | ++ | + | ± | ++ | + | − | − | − | − | − | Cow 1373 |
| 404 | ++ | ++ | + | ++++ | C | ++++ | + | − | − | | Sow 401 |
| | ++ | ++ | ++ | +++ | C | ++++ | − | − | | | Cow 1373 |
| 460 | + | + | ± | + | + | − | − | − | − | | Sow 401 |
| | ± | ± | − | ++ | ++ | − | − | − | | | Cow 1373 |
| 461 | ++ | ++ | +++ | ++++ | C | C | C | ++++ | ++ | | Sow 401 |
| | ++ | ++ | +++ | +++ | C | C | C | ++ | − | | Cow 1373 |
| 462 | ++ | + | +++ | C | C | C | ++++ | +++ | − | | Sow 401 |
| | + | + | +++ | C | C | C | ++++ | + | − | | Cow 1373 |

*The Effect of Feeding Cultures from This Outbreak on Swine.*—Only one experiment has been completed to date.

*Sow 341*, born June, 1925. Last litter May 4, 1927. Late in September the sow was put into an outdoor enclosure provided with a small house for shelter on soil not overrun by swine for at least 13 years. A Blake bottle containing an agar layer was seeded with strain from Swine 401, now 5 months under cultivation, and incubated for 2 days. On Oct. 8, 1927, the sow was fed with the salt solution suspension of the entire culture mixed with dissolved powdered milk.

The sow gave birth to two normal young on October 19. Placental material was not recovered nor any material indicating the birth of dead pigs. The agglutinin reactions of the blood of the sow on the day of feeding and the day following farrowing are given in Table IV.

Following the farrowing, a swab was inserted into the uterus and the attached material suspended in normal saline and injected into the peritoneal cavity of two guinea pigs. Both were chloroformed after 46 days. Autopsy and cultures were negative. At the same time two guinea pigs received an intraperitoneal injection of the sow's milk. Both, killed after 46 days, had large spleens and other lesions diagnostic for *Bacillus abortus* infection. Pure cultures of *Bacillus abortus* were obtained from spleen tissue. The guinea pig lesions

TABLE IV.

*Agglutination Reactions of Sow before and after Infection.*

| Blood drawn | Serum dilutions | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 1/10 | 1/20 | 1/40 | 1/80 | 1/160 | 1/320 | 1/640 | 1/1280 | 1/2560 |
| October 8..... | ++++ | ++++ | C | ++++ | + | ± | − | − | − |
| " 20..... | ++++ | +++ | ++++ | C | C | +++ | + | ± | − |

resembled closely those produced originally by the culture fed. The significant result of this experiment is contained in the fact that the organism fed became located in the mammary gland and not in the uterus of the pregnant sow. The agglutinin test before infection suggests possible infection of the sow with *Bacillus abortus*, but tests on other sows of the herd yielded nearly the same figures. The herd has been free from abortion and recent tests of the milk from four sows on guinea pigs were entirely negative.

In the spring of 1928, the attention of this Department was called to an outbreak of swine abortion in another State institution.[4] The sows gave birth to large litters of which most died on the same day. Material from uterine swabs and sow's milk were injected into a large number of guinea pigs. All of these when killed 4 to 6 weeks later

[4] The writer is indebted to Dr. Henry W. Dustan for material from this outbreak.

were normal and cultures of the entire, small spleen negative. Similarly cultures made from the organs of four new-born pigs were negative.

In this outbreak *Bacillus abortus* evidently played no part. The work is mentioned here so that those entrusted with the investigation of such outbreaks on the spot do not fail to consider causes other than infection with *Bacillus abortus*.

## SUMMARY.

The outbreak of infectious abortion in swine, probably the first reported from the eastern United States, was associated with a strain of *Bacillus abortus* growing rapidly on ordinary nutrient agar slopes without seal and presenting certain slight pathological deviations from the bovine form of disease in guinea pigs such as the occurrence of necrotic, suppurating foci in spleen and lymph nodes. Agglutination tests, comprising both cross-agglutination and absorption procedures failed to distinguish the strain from the bovine type. The gross appearance of the fetuses from this outbreak was normal. The shreds of placentas obtainable indicated slight erosion of the chorionic epithelium and some exudation. The specific bacilli were quite widely disseminated in the tissues of the fetuses. The pathogenic action of this swine strain on guinea pigs was evidently much feebler than that of most earlier swine strains as reported and it approached more closely that of bovine strains. The culture fed to a pregnant sow failed to produce abortion, possibly because of the advanced stage of pregnancy. The organism was not recovered from the uterus but was found in the sow's milk.

# THE REACTION OF HUMAN BILE AND ITS RELATION TO GALL STONE FORMATION.

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Little is known concerning the chemical changes occurring in bile which lead to the formation of calculi, even though the problem of gall stone formation has attracted a great deal of attention over a long period. This absence of knowledge is especially evident with respect to the acid-base equilibrium of the bile. The suggestion has been made repeatedly that the concentration of hydrogen ions may be one of the factors which governs the solubility of calcium and the bile pigments. In addition, some evidence exists that the solubility of the cholesterol is influenced by the reaction of the bile.

Of the few researches that have dealt with the hydrogen ion concentration of bile, only those of Drury, McMaster and Rous (1) and of Stern (2) have considered its possible effect upon gall stone formation. The work of the former confirmed the observations of Okada (3) and of Neilson and Meyer (4) that bile from the gall bladders of animals was more acid than bile which had been freshly secreted by the liver. They found similar differences in human specimens, and observed further that the acidity of dog bile increased progressively with the time of its stay in the gall bladder. A study of the calcium concentration showed that it rose and fell with the hydrogen ion concentration and that calcium carbonate and cholesterol were precipitated from alkaline bile, whereas acidification prevented the precipitation. Neilson and Meyer had previously found that the diseased gall bladder of the rabbit was unable to change the reaction or the concentration of the bile. On the basis of these observations, Drury, McMaster and Rous suggested that one of the functions of the gall bladder was to acidify the bile and in this way increase its solvent power for calcium and perhaps cholesterol. Failure of the gall bladder to function in making the bile more acid,

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particularly while it is being concentrated, presumably would be followed by precipitation and possibly by calculus formation. Recently Drury and Rous (5) attempted to test this hypothesis experimentally by altering the gall bladders of dogs and rabbits so that the bile would not become more acid during its storage in the gall bladder. In one case gall stones formed, although the experiment was complicated by bacterial infection. The work of Stern (2) is based on his study of cholesterol hydrosols. It is his belief that gall stones are formed because of an abnormally increased hydrogen ion concentration in the bile; however, his attempts to form concretions by massive administrations of acid to animals were unsuccessful.

Before either of the hypotheses just described is accepted more direct evidence is needed. In our work, therefore, the hydrogen ion concention of human bile has been studied in order to ascertain whether gall stones are associated with a change in its reaction. In connection with this study additional evidence was obtained relative to the action of the gall bladder in modifying the pH in the bile.

### *Method.*

The specimens were aspirated from the gall bladder *in situ* at operation or immediately after its removal with the clamps left in position on the cystic duct.[1] A syringe was used for withdrawing the specimen. Exposure to air with subsequent loss of $CO_2$ was avoided.

Fistula bile was collected under mineral oil, care being taken to keep the drainage tubes filled with bile and free from air bubbles. It was difficult to do this and several experiments were invalidated because of changes in the bile brought about in this way. Our experience in collecting bile from fistulæ suggests that the more alkaline values commonly reported for fistula bile may be the result of unsuspected losses of $CO_2$ within the drainage system. The use of mineral oil to prevent gas exchange was not satisfactory because the loss of $CO_2$ was not completely prevented, but no better method was available for making

[1] The preoperative treatment of the patients was the same in all cases except one (noted below). A light meal was given at 5 p.m. on the preceding day, following which the patient received no other food until after the operation. An enema was given in the morning and was followed, 30 minutes before operation, by the usual injection of morphine and atropine sulfates. All of the operations were done in the morning, hence the collection of the bile had been preceded by 15 to 19 hours of fasting.

The only deviation from this procedure was made in a case of diabetes (Table II, Feb. 27, 1928) when glucose and orange juice were given with 10 units of insulin 1¾ hours before operation.

continuous collections over many hours. In a few instances the bile was collected in sampling tubes over mercury. By comparing these specimens with some taken under oil, the error caused by the use of the latter was determined and the corresponding correction applied. The maximum change was 0.005 pH per hour of exposure to the mineral oil. The specimens from the gall bladder were tested at once and did not need correction.

The pH was determined by the use of the quinhydrone electrode. When enough material was available the results were checked by means of the hydrogen electrode. The refill technique described by Cullen (6) was used with the latter. Quinhydrone electrode determinations were made by means of the apparatus and procedure described by Meeker and Reinhold (7). Results by the two methods were practically identical. The average of all determinations by the quinhydrone electrode was 0.015 pH less than the average of the hydrogen electrode determinations on the same specimens. The maximum differences were +0.01 pH and −0.05 pH. Some drift of the potentials occurred when the quinhydrone electrode was used in bile, but at a much slower rate than in the case of similar determinations in blood plasma. Error from this source was overcome by reading the potential as quickly as possible after mixing the quinhydrone into the sample. It had been demonstrated previously that this precaution eliminated the error due to drifting when the method was used for plasma pH determinations.

## EXPERIMENTAL DATA.

The hydrogen ion concentration of human bile from the gall bladders of unobstructed, calculus-free biliary systems varied between pH 7.10 and 7.30. The figures are given in Table I. There were no apparent pathological changes except that three of the gall bladders showed adhesions. Possibly it is significant that the bile from gall bladders with adhesions was slightly more alkaline than the other specimens in this group. Two of the gall bladders showing these changes were tested by the Graham method. Both functioned normally, so far as ability to concentrate or evacuate the bile was concerned.

No data for the hydrogen ion concentration of normal human gall bladder bile were discovered in the literature. Labbé, De Moor and Nepveux (8) determined the pH colorimetrically of diluted bile obtained by non-surgical drainage of the gall bladder by means of the Meltzer-Lyon technique. Their specimens varied between pH 6.6 and 7.4. Contamination by the contents of the duodenum was inevitable in samples collected in this way.

The specimens from gall bladders in which calculi were present were classified according to the presence or absence of obstruction of the bile

ducts. The data on unobstructed biliary systems are presented in Table II. With two exceptions the pH values were either above or below the normal limits established in Table I. Four of the fifteen specimens were slightly more alkaline than the highest of the normal group, but a fifth was 0.40 pH more alkaline. Eight were more acid. The differences measured from the lowest normal figure, were 0.01 pH to

TABLE I.

*The Reaction of Human Gall Bladder Bile in the Absence of Concretions or Obstruction.*

| Date | Number | Bile pH 37.5°C. | Culture | Remarks |
|---|---|---|---|---|
| *1927* | | | | |
| Apr. 20 | *10693 | ‡7.19 | | Carcinoma of the stomach |
| May 18 | | 7.24 | | Adhesions around gall bladder |
| Dec. 2 | 12252 | 7.10 | Negative | |
| *1928* | | | | |
| Jan. 15 | 12733 | 7.13 | Negative | |
| Apr. 16 | 13266 | 7.22 | Negative | †Gall bladder gave normal response to test of function. Pericystic adhesions |
| 23 | 13317 | 7.26<br>7.31 | Negative | Normal gall bladder function. Adhesions |

* Index number of the case history in the records of the University of Pennsylvania Hospital.

† Emptying and concentrating power of the gall bladder as determined by the Graham method.

‡ The patients had fasted 15 to 19 hours. The preoperative treatment was the same as in the cases with gall stones or obstruction.

0.22 pH. The direction or the size of the change in reaction was not related to the type of the stone found, nor were there any systematic or consistent differences which could be related to the presence of calculi. In view of the small number of specimens which were obtained from calculus-free gall bladders, some of the preceding probably represent unchanged values. Slight obstruction of the cystic duct, unde-

TABLE II.

*The Reaction of the Human Gall Bladder Bile When Concretions Were Present and Obstruction Was Absent or Slight.*

| Date | Number | Bile pH 37.5°C. | Concretions | | Culture | Remarks |
|---|---|---|---|---|---|---|
| | | | Number | Type* | | |
| *1927* | | | | | | |
| May 27 | 11106 | 7.42<br>7.42 | + | Cholesterol pigment | | Subacute cholecystitis. Acute pancreatitis. Gall bladder function poor |
| June 20 | 11155 | 7.32<br>7.32 | ++ | Cholesterol pigment | Negative | |
| Oct. 19 | 12236 | 6.88<br>6.92 | 24 | Cholesterol pigment | | Chronic interstitial cholecystitis. Gall bladder function normal |
| 28 | 11999 | 6.94 | 8 | Cholesterol pigment | *Staph. hæmolyticus.* Diphtheroids | Chronic interstitial cholecystitis. Cholesterol deposits on gall bladder wall |
| Nov. 2 | 12051 | 7.26<br>7.33<br>7.34 | 20 | Cholesterol pigment | | Chronic interstitial cholecystitis. Cholesterol deposits. Adhesions to duodenum |
| 14 | 12263 | 7.04†<br>7.06† | 1 | Cholesterol pigment | Negative | Acute hemorrhagic cholecystitis. Edema |
| Dec. 2 | 12266 | 6.97<br>6.97 | +++ | Cholesterol | Diphtheroids | Acute pancreatitis. Gall bladder normal |
| *1928* | | | | | | |
| Jan. 15 | 12563 | 7.18 | 16 | Cholesterol pigment | | Chronic interstitial cholecystitis |
| Feb. 27 | 13134 | 7.09 | 1 | Cholesterol (crystals). No pigment | *Staph. albus* non-hemolytic | Cholesterol deposits on gall bladder walls. Function poor |

* The classification of calculi in this and other tables is made on the basis of physical appearance. Chemical analyses were not made.

† Contaminated with blood.

TABLE II—*Concluded.*

| Date | Number | Bile pH 37.5°C. | Concretions | | Culture | Remarks |
|---|---|---|---|---|---|---|
| | | | Number | Type | | |
| *1928* | | | | | | |
| Feb. 27 | 13034 | 7.00 | 40–50 | Cholesterol pigment | Negative | Chronic interstitial cholecystitis. Gall bladder nearly full of stones |
| Mar. 29 | 13044 | 6.99 | + | Calcium pigment | Negative | Gall bladder had normal appearance |
| 30 | 13261 | 7.07 | 11 | Cholesterol | Hemolytic *Staph. aureus* | Subacute interstitial cholecystitis. Cholesterol deposits in gall bladder |
| Apr. 22 | 13708 | 7.68<br>7.70 | ++ | Cholesterol pigment | | Subacute cholecystitis |
| May 14 | | 6.95<br>6.96 | ++ | Cholesterol | Negative | Gall bladder apparently normal |
| June 6 | | 7.35 | + | | Negative | Chronic cholecystitis |

tected at operation, may have existed in others and must also be considered in explaining these observations.

In the presence of obstruction the acidity of the gall bladder contents was increased (Table III). Complete obstruction of the common or cystic duct was characterized by pH values near 6.40. When the obstruction was only partial the pH was higher. An exception occurred in a case of obstruction caused by a carcinoma of the head of the pancreas (Table III, July 8), in which the gall bladder was almost completely atrophied. The specimen, taken from the greatly enlarged common duct, was pigment-free. Its pH was 7.30. Apparently the obstructed bile was not acidified by the liver; otherwise in this instance the usual fall in pH would have occurred. Instead it was dependent on the presence of the gall bladder and must have happened within the latter organ. Neither was the increased acidity a result of mechanical irritation by concretions in the gall bladder. Table III

TABLE III.

*The Reaction of Bile from the Human Gall Bladder Following Obstruction of the Common or of the Cystic Ducts.**

| Date | Number | Bile pH 37.5°C. | Concretions | | Culture | Remarks |
|---|---|---|---|---|---|---|
| | | | Number | Type | | |
| *1927* | | | | | | |
| June 27 | 11338 | 6.37<br>6.40 | 3 | Cholesterol pigment | Negative | Pigment-free "white bile." Cystic duct obstruction. Acute cholecystitis |
| July 8 | 11291 | 7.30 | 0 | | | Carcinoma of head of pancreas. Gall bladder atrophied. Specimen taken from cystic duct. Pigment-free |
| Nov. 8 | 12068 | 6.82<br>6.83 | + | Cholesterol pigment | *B. coli communis* | Acute cystic duct obstruction. Marked edema. Specimen practically colorless |
| 17 | 12126 | 6.44 | + | Cholesterol pigment | Diphtheroids. *Staph. albus* | Chronic cholecystitis. Complete obstruction. Specimen was colorless |
| 16 | 12340 | 6.77<br>6.79 | 40 | Cholesterol pigment | Negative | Subacute interstitial cholecystitis. Partial obstruction |
| 16 | 12135 | 6.70 | + | Cholesterol pigment | *B. coli communis* | Nearly complete obstruction. Edema. Bile contaminated slightly with pus |
| 30 | 12257 | 6.79 | 2<br>Many crystals | Cholesterol | Negative | Acute cholecystitis Partial edematous obstruction |
| *1928* | | | | | | |
| Feb. 22 | 12830 | 6.61 | 1 | Soft sediment. Cholesterol crystals, pigment | *B. coli communis* | Chronic and acute cholecystitis. Thickened diseased gall bladder with adhesions. Partial obstruction of cystic duct |

* Unless stated to the contrary the site of obstruction was in the common duct.

TABLE III—*Concluded.*

| Date | Number | Bile pH 37.5°C. | Concretions | | Culture | Remarks |
|---|---|---|---|---|---|---|
| | | | Number | Type | | |
| *1928* | | | | | | |
| Mar. 7 | 13092 | 6.43 | 4 | Cholesterol | Negative | Chronic interstitial cholecystitis. Partial cystic duct obstruction. Gall bladder walls thickened. Adhesions. Pale yellow bile |
| 23 | 13098 | 6.70 | 2 | Cholesterol | | Acute cholecystitis. Incomplete obstruction. Bile had light green color |
| 29 | | 6.60 | + | Tar-like sediment | Negative | Carcinoma of papilla of Vater. Nearly complete obstruction. Yellow incrustation on gall bladder wall. Yellow bile, low pigment concentration |
| Apr. 23 | 13578 | 6.40 | 12 | Cholesterol pigment | *B. coli communis* | Complete obstruction of cystic duct |
| June 4 | | 6.87 | 36 | Calcium pigment | Negative | Partial obstruction of cystic duct. Thickened gall bladder wall with lesions |

includes a case (Mar. 29), in which the common duct was blocked by a carcinoma of the papilla of Vater. The gall bladder appeared to be normal and no concretion was present, although a small amount of sediment had collected in the fundus. The pH of this bile was 6.60, a figure which corresponded well with those obtained when the same degree of obstruction had been brought about by gall stones.

The result of the obstruction so far as change in the acidity of the bile was concerned, did not differ when the site of the obstruction was in the cystic duct. In either event the effect would be to oppose the expul-

sion of the contents of the gall bladder while interfering to a lesser extent with the flow of bile into the gall bladder. As a consequence the time during which the bile was acted upon would be lengthened and changes in composition accentuated. The higher acidity of the contents of the completely obstructed gall bladder, therefore, can be explained best as being a result of the prolonged exposure to the action of this organ. These specimens were characterized by low concentrations of pigment and solids. The viscosity was greatly increased.

The acidification of the bile was not related to the presence of microorganisms. More than 50 per cent of the pathological specimens were sterile, yet gave results identical with those obtained when infected specimens were studied.

*Fistula Bile.*—Additional evidence concerning the changes in the reaction of human bile was secured by collecting simultaneously samples of bile from the gall bladder and from the common duct. Bile from the gall bladder was obtained from a drainage tube inserted into the fundus. Common duct bile was collected from a common duct fistula.[2]

A sufficient number of specimens was obtained to show that after an initial period of recovery from the operation, the bile from the gall bladder was definitely more acid than that from the common duct (Tables IV and V). Shortly after the operation the difference was small or non-existent. As the condition of the patient returned toward normal, the bile from the gall bladder remained at the same pH or increased slightly, while the pH of the common duct bile increased appreciably. Finally when a light general diet had been resumed and recovery was well under way, the bile from the former was more acid than the latter by from 0.04 to 0.24 pH. Because of the smaller output of the gall bladder fistulæ and the longer drainage tubes required, the probability of error due to loss of $CO_2$ was greater for these specimens than for those from the common duct fistulæ. Consequently such errors would diminish the difference between the hydrogen ion concentrations of the bile from the two sources and would tend to obscure the changes which had taken place.

[2] The collection of bile was made under approximately sterile conditions. Together with the short intervals at which the specimens were collected this should have eliminated any significant error which might have been caused by bacterial action.

TABLE IV.

*The Reaction of Bile Collected Simultaneously from Common Duct and Gall Bladder Fistulæ.*

| Date | Time | Gall bladder bile | | Common duct bile | | | Diff. | |
|---|---|---|---|---|---|---|---|---|
| | | Volume | pH | Volume | pH | Av. pH for 24 hrs. | pH | |
| *1927* | *hrs.* | *cc.* | *38°C.* | *cc.* | *38°C.* | | | |
| Nov. 16 | | | 6.78 | | | | | *No 12340 Liquid diet |
| 17–18 | 12† | | | 145 | 6.86 | | | Night |
| 18 | 12 | | | 140 | 6.89 | | | Day |
| 18–19 | 12 | 68 | 6.95 | 130 | 6.90 | 6.92 | +0.03 | |
| 19 | 12 | | | 117 | 6.95 | | | |
| 19–20 | 12 | 65 | 6.96 | 85 | 6.95 | 7.05 | −0.09 | |
| 20 | 12 | | | 146 | 7.11 | | | |
| 20–21 | 12 | 68 | 6.96 | 132 | 7.06 | 7.14 | −0.18 | Soft diet |
| 21 | 12 | | | 188 | 7.19 | | | |
| 21–22 | 12 | 85 | 7.00 | 159 | 7.19 | 7.24 | −0.24 | |
| 22 | 12 | | | 219 | 7.27 | | | |
| 22–23 | 12 | | 7.08 | 85 | 7.26 | 7.23 | −0.15 | Drainage tubes clamped 12 hrs. Plasma pH 7.39 |
| 23 | 12 | | | 155 | 7.22 | | | |
| 23–24 | 12 | | 7.08 | 107 | 7.23 | 7.24 | −0.16 | Drainage tubes clamped 12 hrs. |
| 24 | 12 | | | 65 | 7.26 | | | |
| 25 | 12 | 53 | 7.14 | 58 | 7.35 | 7.35 | −0.21 | Drainage tubes clamped 12 hrs. Plasma pH 7.38 |
| 25–26 | 12 | | | 45 | 7.35 | | | |
| 28 | ½ | | | | 7.27 | | | Before breakfast |
| | 2½ | | | | 7.43 | | | After breakfast |
| | ½ | | | | 7.31 | | | After preceding |
| 29 | ½ | | | | 7.37 | | | Before breakfast |
| | 5 | | | | 7.35 | | | After breakfast |
| | ½ | | | | 7.39 | | | After preceding |

* See Table III, Nov. 16.

† The specimens were collected over intervals varying between 2 and 6 hours. In order to save space and to make the data more accessible the results have been expressed in terms of 12 hour periods beginning at 8 a.m. and 8 p.m. The same applies to Table V.

The ingestion of a test meal in one instance was followed by an alkaline tide in the bile during a $2\frac{1}{2}$ hour period. A similar experiment on the following day, in which the collection of the postprandial specimen was extended over 5 hours, failed to show a significant change.

TABLE V.

*The Reaction of Bile from Common Duct and Gall Bladder Fistulæ.*

| Date | Time | Gall bladder bile | | Common duct bile | | | Diff. | |
|---|---|---|---|---|---|---|---|---|
| | | Volume | pH | Volume | pH | Av. pH for 24 hrs. | pH | |
| *1927* | *hrs.* | *cc.* | *38°C.* | *cc.* | *38°C.* | | | |
| Nov. 17 | | | 6.44 | | | | | *No. 12126 |
| 19 | 12 | | | 60 | 6.83 | | | Day |
| 19–20 | 12 | | | | 6.81 | | | Night |
| 20 | 12 | | | 215 | 7.01 | | | Liquid diet |
| 20–21 | 12 | | | 140 | 6.96 | | | |
| 21 | 12 | 11 | 6.94 | 155 | 6.97 | 7.00 | −0.06 | |
| 21–22 | 12 | | | 139 | 7.03 | | | |
| 22 | 12 | 61 | 6.97 | 95 | 7.03 | 7.07 | −0.10 | Soft diet |
| 22–23 | 12 | | | 150 | 7.10 | | | |
| 23 | 12 | 21 | 6.97 | 123 | 6.94 | 7.01 | −0.04 | Drainage tubes clamped 6 hrs. |
| 23–24 | 12 | | | 171 | 7.06 | | | |
| 24 | 12 | | | 153 | 7.03 | | | Plasma pH 7.34 |
| 24–25 | 12 | | | | 7.06 | | | |
| 25 | 12 | | 6.98 | 75 | 7.06 | | −0.08 | |

* See Table III, November 17.

The reaction of the common duct bile in the few comparisons made was slightly more acid than that of the blood.

## DISCUSSION.

Under certain conditions marked changes occurred in the hydrogen ion concentration of the gall bladder bile. From the data presented

it is apparent that these were not associated with the presence of concretions in the gall bladder. While deviations from the "normal" reaction were observed in almost every instance when gall stones were present, it seemed improbable that these changes were of sufficient size to influence solubility relationships. However, it was possible that the formation of calculi was caused by transitory changes in the acid-base balance of the bile or by a succession of such changes. It would then be unlikely that more than a few of the specimens in Table II would vary much from the normal values. Actually only one specimen showed a large difference, although six others differed from the lowest or highest normals by more than 0.10 pH. The acid character of several specimens of this group was probably the result of the same causes which increased the acidity of the bile during obstruction.

The pronounced increase in the acidity of bile following obstruction of the ducts coincided with the observations made by Drury, McMaster and Rous (1) on the pH of the gall bladder bile of dogs in which stasis had been induced experimentally. Obstruction increased the time during which the bile was exposed to the action of the gall bladder. If the latter was able to acidify its contents, those specimens which had been exposed to its action for a long time would necessarily have become more acid than those which had been expelled with less delay. Consequently complete obstruction was accompanied by the greatest acidification. Several additional facts supported the conclusion that it was the gall bladder which brought about the change in the reaction of the bile. Most conspicuous was the failure of the biliary system to acidify the bile when the gall bladder was absent (atrophied). Of even greater significance was the consistently higher hydrogen ion concentration in bile from a gall bladder fistula when compared with that from a common duct fistula, collected over the same time interval. That the changed acidity was not the result of the irritating action of the calculi was shown by the occurrence of identical changes in obstructed calculus-free gall bladders. Infection was ruled out by similar reasoning.

While it seemed quite certain from the foregoing that the gall bladder altered the hydrogen ion concentration of the bile, this should not be allowed to obscure the possibility that the acid bile found in the gall bladder following obstruction was secreted as such by the liver either

as a result of damage to that organ or in an attempt to increase the solvent power of the bile. The acidity of bile coming through a common duct fistula from the liver was greatest after severe obstruction of the common duct, and some of this acid secretion must have entered the gall bladder. In cystic duct obstruction, however, there was no injury to the liver and the secretion and composition of bile from the liver presumably was not affected. Since the change in reaction of the gall bladder contents was the same in direction and degree after obstruction of the cystic duct as it was after common duct obstruction, the changes which occurred must have been accomplished by the gall bladder.

No evidence is available concerning the means by which the contents of the gall bladder are made acid. Absorption of material from the bile by this organ as shown by a decrease in solids (unpublished data) is one probability, while secretion by the gall bladder as reflected by the increased viscosity of the contents likewise may have had a part in the changes which occurred. Whatever the mechanism, it is apparent that even marked pathological changes did not prevent it from functioning. The significance of the variations in the hydrogen ion concentration of the bile remains obscure. It is hoped that solubility studies now in their preliminary stages in this laboratory will assist in establishing their relation to calculus formation.

## SUMMARY.

1. The human gall bladder acidifies the bile. In this respect its action is similar to that of the gall bladders of lower animals, previously described by other workers.

2. The hydrogen ion concentration of gall bladder bile is increased considerably in cases of obstruction of the common or cystic ducts. The highest values were found following complete obstruction.

3. The occurrence of gall stones was not associated with a consistent change in the hydrogen ion concentration of the gall bladder bile.

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# DIFFERENTIATION BETWEEN SOME TOXIC SUBSTANCES IN ANAEROBICALLY PRODUCED AUTOLYSATES OF PNEUMOCOCCI (TYPES I AND II).

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(Received for publication, January 22, 1929.)

In preceding papers (1, 2), it was shown that anaerobically produced autolysates of pneumococci cause necrosis when injected intradermally, and marked lung lesions and death when injected intratracheally, into small guinea pigs. In the present study, I wish to describe experiments made to determine whether or not the necrotizing and the lung-toxic effects are due to the same or to different substances present in these autolysates.

## EXPERIMENTAL.

### *Preparation of the Poisonous Autolysates.*

The method of preparation of the toxic autolysates has been described in detail in previous papers (1, 2). Briefly, the method is to autolyze in fresh broth at room temperature under vaseline seal, the centrifuged pneumococci obtained after 18 hours growth on double strength veal infusion broth which contains 4 per cent Witte peptone. The Berkefeld filtrates of these autolysates are toxic for both the skin and the lungs of young guinea pigs and both these toxic effects can be preserved without deterioration when such filtrates are kept in the ice box under vaseline seals.

### *Heat Lability of the Toxic Autolysates for the Lungs of Guinea Pigs.*

In a previous article it was shown that the necrotizing principle was completely destroyed when heated to 60° for 5 minutes under vaseline seal. It was found that under these same conditions the lung-toxic principle was also destroyed. Guinea pigs injected intratracheally with 0.2 cc. of the heated autolysate did not appear sick at any time and did not die spontaneously. Several of these pigs were

killed with ether 1 to 3 days after the injection of heated poison, and all showed at autopsy a small red patch of consolidation in one or more lobes of the lungs near the hilum, but never the diffuse lesion caused by the unheated autolysates.

*Sensitiveness to Oxidation of the Toxic Autolysates for the Lungs of Guinea Pigs.*

It was noted in a previous paper that the skin-necrotizing poison in the autolysates was extremely sensitive to oxidation and deteriorated rapidly when exposed to the air. We found that the lung-toxic substance also weakened quickly after removal of the vaseline seal, even when the tubes containing the preparations were kept in ice water. A few experiments were made with aerobically prepared autolysates,—autolysates prepared exactly like the anaerobic autolysates, except that the pneumococci were autolyzed without the vaseline seal and the filtrates preserved in the ice box were also without a vaseline seal. These aerobically prepared autolysates produced no skin reaction when injected intradermally and no symptoms or death when injected intratracheally into young pigs. In some pigs killed after the injection of aerobic autolysates, small areas of consolidation near the hilum were noted, similar to those found after the injection of heated autolysates.

*Is the Lung-Toxic Product Neutralizable by the Anti-Autolysate Serums Which Neutralize the Necrotizing Principle?*

It was shown previously that there was an apparent neutralization of the necrotizing principle when necrotizing autolysates from either Pneumococcus I or II were mixed *in vitro* with the serum of rabbits immunized against Pneumococcus I autolysates. It was important to determine whether the substance or substances which were responsible for the death of guinea pigs injected intratracheally with the toxic filtrates could also be neutralized by the antiserums which neutralized the necrotizing principle.

The tests for neutralization of the lung-toxic product were set up in a way similar to our former tests for neutralization of necrotizing substances. These tests were carried out as follows: 0.9 cc. of a well chilled necrotizing filtrate was placed in

each of three narrow test-tubes. To the first tube was added 0.1 cc. of the anti-serum; to the second tube 0.1 cc. of normal rabbit serum; and to the third the same amount of broth. The contents of the tubes were well mixed, and a heavy vaseline seal was then added to each tube. After standing at room temperature for 1 hour, the tubes were again chilled, the vaseline seals removed, and the preparations in amounts of 0.25 cc. were injected intratracheally into small guinea pigs of approximately equal weights. The results of these experiments were clear-cut. The pigs which received the toxic autolysate and immune serum showed no symptoms and survived. The other two pigs died with the usual symptoms and the autopsy findings were typical. This experiment has been repeated several times and always with the same results.

TABLE I.

| | Toxicity for guinea pigs' lungs. 0.2 cc. injected intratracheally | Toxicity for guinea pigs' skin. 0.1 cc. injected intracutaneously |
|---|---|---|
| Necrotizing filtrate | +++ | +++ |
| Adsorbed with red cells | 0 | +++ |

These experiments have been repeated with two potent anti-autolysate horse serums. These serums in amounts of 0.0002 cc. neutralized at least one lethal dose of the lung-toxic autolysates, *viz.*—0.1 cc. of these serums diluted 1–100 neutralized 0.9 cc. or more than five lethal doses of the toxin. (These last experiments will be given in detail in a later publication.)[1]

From the fact that the anti-autolysate serum prepared with Type I neutralized the lung-toxic action of the autolysates of both Pneumococcus Type I and Type II, it is evident that the lung-toxic poison, like the pneumococcus necrotizing poison, is antigenically similar for both Types I and II and is therefore not type-specific.

These experiments seem to show that the necrotizing and lung-toxic poisons in the anaerobic autolysates are similar in their sensitiveness to heat and to oxidation and in their ability to be neutralized by the same anti-autolysate serums.

[1] These horses were immunized to the autolysate filtrates at Eli Lilly Co., in Indianapolis, under the supervision of Mr. W. A. Jamieson.

*Toxicity of Red Cell-Adsorbed Autolysates for the Lungs of Guinea Pigs.*

In a preceding paper, it was shown that necrotizing filtrates from which the hemotoxin had been removed by adsorption with red cells, were just as toxic for the skin of guinea pigs as untreated autolysates. It seemed of interest to determine whether or not such treatment had any effect on the toxicity of these autolysates for guinea pig lungs.

Accordingly guinea pigs of approximately equal weight were injected intratracheally with 0.2 cc. of necrotizing filtrates or the same filtrates which had just previously been adsorbed with red cells. Six different autolysates were tested in this manner. All the guinea pigs which received the untreated autolysates died with characteristic symptoms and autopsy findings, while none of the pigs which were inoculated with adsorbed autolysates showed any symptoms or died. Five of the latter pigs were killed with ether from 1 to 3 days after the injection, and at autopsy showed very much the same picture grossly as the pigs which had been injected intratracheally with the heated toxin described before. None showed the diffuse pneumonia regularly found in control animals. The consolidation about the hilum was, however, more extensive than in the pigs given heated toxin. All autolysates, both treated and untreated, were tested each time for necrotizing activity by inoculation into guinea pigs' skin and always proved to be equally toxic for the skin. These last experiments are summarized in Table I.

It is evident from these experiments that the substance or substances in the necrotizing autolysates, which when injected intratracheally bring about marked symptoms and death of guinea pigs, had been removed or inactivated by treatment with the red cells. An important question to be answered was whether or not the toxic substance for the lungs which had been removed was the pneumococcus hemotoxin, which is known to be adsorbed by red cells.

*Toxicity of Pneumococcus Hemotoxin for the Lungs of Guinea Pigs.*

To investigate this point, we produced pneumococcus hemotoxin by the freezing and thawing method of Avery and Neill (3) and inoculated the filtrates of these extracts, which were strongly hemolytic, intratracheally into guinea pigs. As a general rule, these hemotoxin preparations caused no symptoms and only slight lung involvement when injected intratracheally. However, we have obtained two preparations which were toxic for the lungs of guinea pigs, causing a diffuse lesion similar both macroscopically and microscopically, to that

caused by a weak lung-toxic autolysate. It is probable, therefore, that the pneumococcus extracts do occasionally contain the lung-toxic poison in considerable amounts. That the lung-toxic action of our autolysates is not due to the hemotoxic properties of the hemotoxin itself seems probable from the fact that no evidence of hemolysis was seen in the sections of the lungs of the pigs injected with the toxic autolysates and that the lung-toxic action of an autolysate may be neutralized by quantities of anti-autolysate serums which have no effect on the hemotoxin. (This last point will be brought out in greater detail in a later paper.) We have also found no definite parallelism in our autolysates between the toxic action for lungs of

TABLE II.

| Toxic products in anaerobic autolysates | Present in aerobic autolysates | Present in anaerobic autolysates | Present in frozen and thawed pneumococcus extracts | Present in anaerobic autolysates adsorbed with red cells |
|---|---|---|---|---|
| Hemotoxin | 0 | + | + | 0 |
| Skin-toxic | 0 | + | 0 | + |
| Lung-toxic | 0 | + | ± | 0 |

guinea pigs and the amount of hemotoxin such autolysates contain. We think it probable, therefore, that the pneumococcus hemotoxin present in the autolysates is not responsible for either the dyspneic symptoms, or the death with marked lung lesions of guinea pigs injected intratracheally with the toxic autolysate, but that preparations of hemotoxin, produced by the freezing and thawing method, may contain definite amounts of the lung-toxic product.

It seems clear, therefore, that although the necrotizing and lung-toxic principles in the autolysates are similar in several respects, they differ in the important point that the necrotizing poison is not inactivated or adsorbed by red cells as is the lung-toxic principle. This fact appears to prove that the necrotizing and lung-toxic principles are separate entities. These facts are summarized in Table II.

## CONCLUSIONS.

The necrotizing and lung-toxic principles present in certain anaerobically prepared autolysates of Pneumococcus Types I and II are similar in respect to extreme sensitiveness to heat and to oxidation, and to their ability to be neutralized by the same anti-autolysate serums.

These two poisons differ, however, in their ability to be adsorbed or inactivated by red cells; the lung-toxic principle being adsorbed or inactivated by such procedure while the necrotizing principle is not.

Since pneumococcus hemotoxin is present in the anaerobic autolysates and is also adsorbed by red cells, it seemed possible that it was this substance in the autolysates which caused the diffuse lung lesions and death of guinea pigs. However, it was found that the intratracheal injection of pneumococcus hemotoxin prepared by the method of Avery and Neill only occasionally produced the characteristic reaction caused by the intratracheal injection of the anaerobic autolysates. From these experiments we believe, therefore, that the necrotizing and lung-toxic principles, and probably the pneumococcus hemotoxin also, are all separate entities in the anaerobically produced autolysates described.

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# SURVIVAL OF THE VIRUS OF POLIOMYELITIS FOR EIGHT YEARS IN GLYCEROL.

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(Received for publication, December 7, 1928.)

The occurrence of epidemics of poliomyelitis raises the question of the length of time which the etiological agent or virus of the disease will survive outside of the animal body. The power of this virus to resist adverse conditions is of interest not only because of its relation to the natural history of the disease, but also in view of the possible application of such information to experimental methods. It was therefore considered worth while to record an instance of the survival of poliomyelitis virus for 8 years in glycerol, particularly since the infectivity of the material remained almost unchanged during this period.

The mechanism responsible for the epidemic nature of the disease has aroused considerable speculation. Flexner and Amoss (1) have pointed out the great variation in the infecting power of a strain of virus much used in experimental work. They (2) suggested that the ability of the virus to survive a long time in mild antiseptics might indicate that a recrudescence of poliomyelitis in a given locality was due to renewed activity of virus which had been dormant since a previous epidemic.

Many attempts have been made to immunize monkeys against poliomyelitis by the use of attenuated virus. The methods used to decrease the infectivity of the material, while preserving its antigenic power, have usually been based on the use of chemical or physical agents, such as phenol, glycerol, heat, and drying. The results have almost always been disappointing, due to the fact that a certain number of animals contracted the disease during the treatment designed to immunize them against it. Such failures were probably caused by the extraordinary resistance shown by some samples of

virus. The persistence of infectivity after long exposure to glycerol is an indication of the hazard of attempts to immunize human beings by the use of virus attenuated by the ordinary methods.

The possibility of exposing poliomyelitis virus to mild antiseptics over long periods is important also from its application to the views of those investigators who consider that the disease is due to a streptococcus. Although Long (3) has cultivated streptococci from pieces of brain tissue which had been kept in 50 per cent glycerol for 303 days, it seems improbable that the organisms could survive in the chemical for a period of 8 years. Needless to say, the material used by us was cultured and streptococci proved absent before it was inoculated into animals.

The first report of the survival of poliomyelitis virus in glycerol is that of Flexner and Lewis (4), published in 1910. These investigators kept the material in 50 per cent glycerol for 7 days and then found it infectious for a monkey. In the same year, Römer and Joseph (5) stated that they had produced poliomyelitis in monkeys with material which had been kept in 100 per cent glycerol for 5 months. 4 years later, in 1914, Flexner, Clark, and Amoss (6) described an experiment in which spinal cord was kept 25 months in 50 per cent glycerol, after which it produced the disease when inoculated in a monkey. Flexner and Amoss (7), in 1917, recorded the successful transfer of virus which had been in glycerol for 6 years. In this case, repeated inoculations of large doses were required to infect the animal, and the authors felt that there had been a definite decline in virulence of the virus.

In the course of investigations on poliomyelitis at The Rockefeller Institute, specimens of the central nervous systems of monkeys which succumb to the disease are removed under rigid aseptic precautions, cut in pieces about 1 cm. in diameter, and placed in a considerable volume of 50 per cent glycerol. Tightly stoppered glass bottles are used and the stopper covered with tin-foil to prevent evaporation. The specimens are kept in the refrigerator at a temperature of approximately 4°C. The glycerol used is free from oxalates and sulfates.

The material employed in the following experiment was a mixture of two strains (M. A. and K.) which had been used for a long time to produce experimental poliomyelitis in monkeys (8). The animals from which the virus was obtained were prostrate on or before the 7th day after inoculation. Spinal cords from seven different monkeys were pooled before grinding and inoculating.

*Experiment 1.*—October 31. Monkey (*Macacus rhesus*) inoculated intracerebrally[1] with 1.5 cc. of a 20 per cent suspension of pooled 1920 mixed virus. November 8, excitement, tremor, and well marked ataxia were noted, with a tendency to fall when jumping about the cage. There was weakness of arms and legs, with inability to raise the arms above the head. November 9, the animal was prostrate, unable to move either arms or legs, and the respiration was barely perceptible. The monkey was killed by ether and the central nervous system removed under aseptic precautions.

Macroscopic examination: The cord was rather soft and edematous. Tiny, punctate, hemorrhagic areas were scattered through the cord and medulla.

Microscopic examination: The meninges showed a slight infiltration with lymphocytes and endothelial cells. Perivascular infiltration with lymphocytes was noted in the cord and medulla. Various degrees of degeneration of nerve cells of the cord were seen, particularly marked in the anterior horns. Here many examples of active neuronophagia were found, and in some places a polymorphonuclear reaction was present around necrotic nerve cells. Some increase in glia elements was noted. The intervertebral ganglia showed diffuse lymphocytic infiltration and some nerve cell degeneration.

*Experiment 2.*—November 9. Monkey (*Macacus rhesus*) was inoculated intracerebrally with 1 cc. of a fresh 5 per cent suspension of spinal cord and medulla of the previous monkey. November 12, the animal was slow in its movements about the cage, was somewhat excited, and showed definite ataxia. November 13, weakness of both arms and legs was observed, and the ataxia was more marked. November 14, the condition was slightly more marked. November 15, there was little change. November 16, the animal was much more excited and weaker, and both arms were paralyzed. November 17, the monkey was found prostrate and moribund. It was promptly killed with ether and an autopsy performed.

Macroscopic examination: The cord was somewhat injected and bulged from edema when cut. Scattered, small, soft hemorrhagic areas were found unevenly distributed throughout its length and the medulla.

Microscopic examination: Fairly well marked lymphocytic reaction was present in the meninges. The blood vessels were congested. Perivascular infiltration with lymphocytes was present throughout the cord and medulla. The nerve cells of the cord showed marked degenerative changes, ranging from slight to complete dissolution. A sharp reaction of lymphocytes and mononuclear cells about the degenerated nerve cells was present. The intervertebral ganglia showed diffuse lymphocytic infiltration.

The virus was passed through three more animals in series, with gradually decreasing doses. All the animals were prostrate within 8 days, even when injected with a dose as small as 0.2 cc. of a Berkefeld filtrate of a 5 per cent suspension. The gross and microscopic changes, as well as the symptoms produced, were in all instances characteristic.

[1] All monkey inoculations were carried out under ether anesthesia.

## DISCUSSION.

In a paper by Flexner and Amoss, in which the successful transmission of poliomyelitis material kept for 6 years in glycerol was described, appreciable difficulty was experienced in obtaining infection in the first monkeys inoculated. A considerable length of time intervened between the introduction of the nervous tissues and the development of symptoms. In several instances, repeated injection of virus was required to produce the disease. These facts were recorded in order to emphasize the long survival as well as reduction in activity of the virus following the long glycerolation. In view of the results described in this paper, in which the virus has been shown to maintain maximal potency after glycerolation for 8 years, the attenuating action of the chemical may well be questioned. That the specimens of virus should have retained the original high infectivity so that on the first inoculation of the glycerolated tissues the monkeys should have developed typical symptoms and lesions of experimental poliomyelitis is not only an unusual occurrence but perhaps one of epidemiological significance.

## CONCLUSIONS.

1. An instance of successful inoculation of poliomyelitis virus after preservation for 8 years in 50 per cent glycerol is reported.

2. The virulence of the material injected remained essentialiy unchanged during this period.

3. The fact that poliomyelitis virus will survive in glycerol for so great a period may be taken as further indication of the improbability of streptococci as the inciting organisms.

4. Poliomyelitis virus would seem to vary in its resistance to glycerolation.

5. The remarkable persistence of active virus outside of the body may have a bearing on the epidemiology of poliomyelitis.

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